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Ririe et al.

(10) **Patent No.: US 8,940,526 B2**
(45) **Date of Patent: Jan. 27, 2015**

(54) **SELF-CONTAINED BIOLOGICAL ANALYSIS**

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(73) Assignee: **BioFire Diagnostics, LLC**, Salt Lake City, UT (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/765,249**

(22) Filed: **Feb. 12, 2013**

(65) **Prior Publication Data**

US 2013/0157349 A1 Jun. 20, 2013

Related U.S. Application Data

(63) Continuation of application No. 11/913,120, filed as application No. PCT/US2006/017665 on May 8, 2006, now Pat. No. 8,394,608.

(60) Provisional application No. 60/679,052, filed on May 9, 2005.

(51) **Int. Cl.**
C12M 1/34 (2006.01)
C12Q 1/68 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC ***C12Q 1/6844*** (2013.01); ***B01L 3/50273*** (2013.01); *B01L 3/502723* (2013.01); *B01L 7/52* (2013.01); *B01L 2200/10* (2013.01); *B01L 2300/0816* (2013.01); *B01L 2300/0867* (2013.01); *B01L 2300/087* (2013.01); *B01L 2300/1822* (2013.01); *B01L 2400/0478* (2013.01); *B01L 2400/0481* (2013.01); *B01L 2400/049* (2013.01); *B01L 2400/0644* (2013.01); *B01L 2400/065* (2013.01);
(Continued)

(58) **Field of Classification Search**
None
See application file for complete search history.

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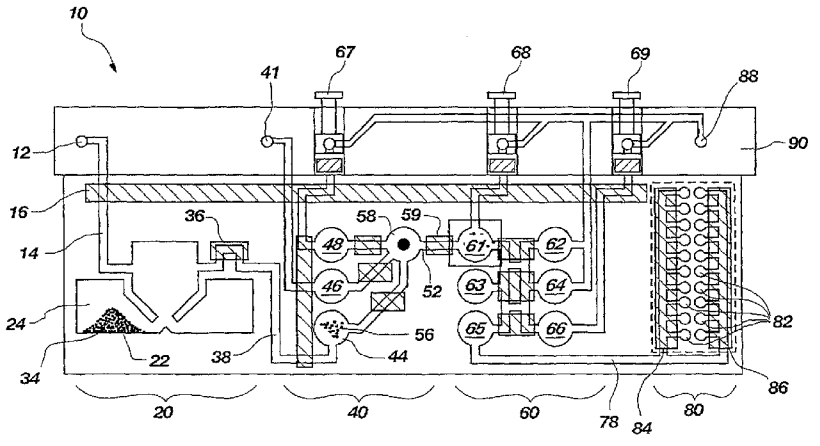
Primary Examiner — Suchira Pande
(74) *Attorney, Agent, or Firm* — Myers Bigel Sibley & Sajovec, P.A.

(57) **ABSTRACT**

Devices, containers, and methods are provided for performing biological analysis in a closed environment. Illustrative biological analyzes include nucleic acid amplification and detection and immuno-PCR.

13 Claims, 12 Drawing Sheets



(51) **Int. Cl.**
B01L 3/00 (2006.01)
B01L 7/00 (2006.01)

(52) **U.S. Cl.**
CPC . *B01L 2400/0655* (2013.01); *B01L 2400/0677* (2013.01); *B01L 2400/0683* (2013.01)
USPC **435/287.2**; 435/289.1; 435/303.1

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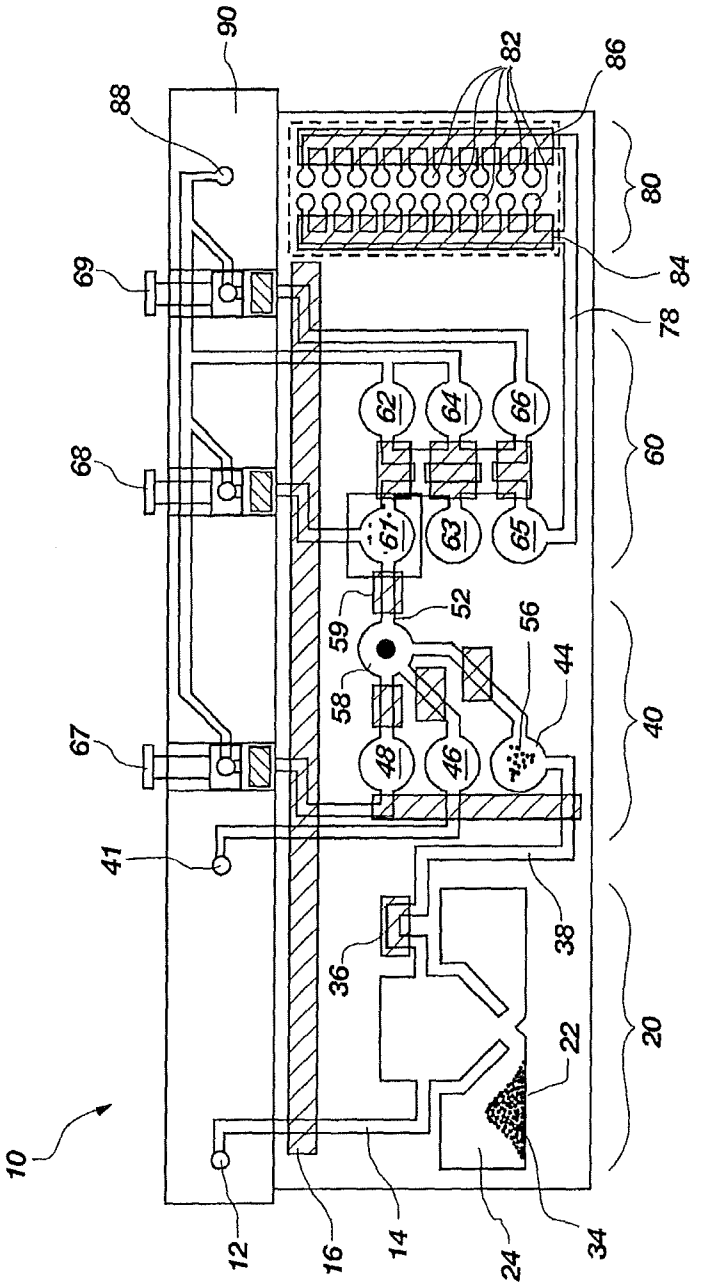
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FIG. 1

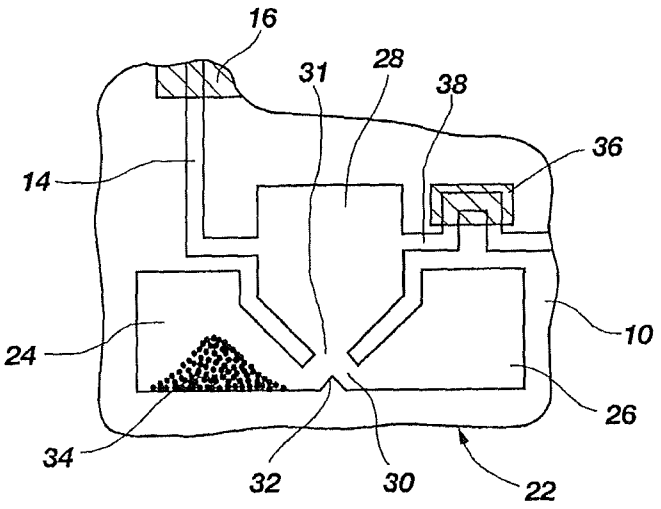
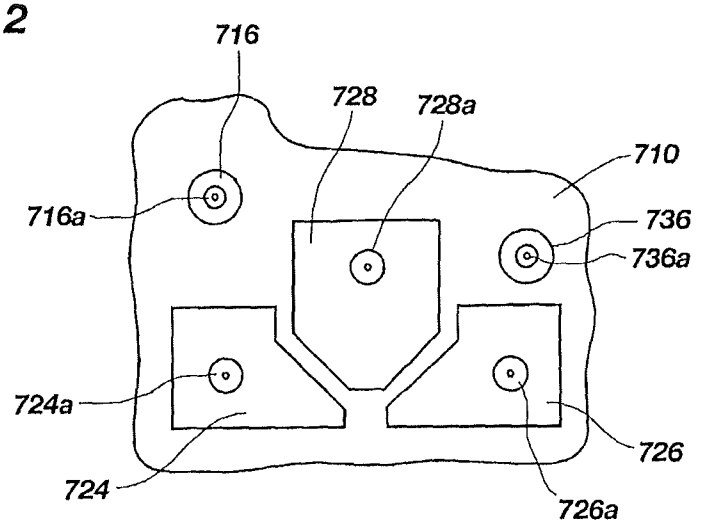


FIG. 2



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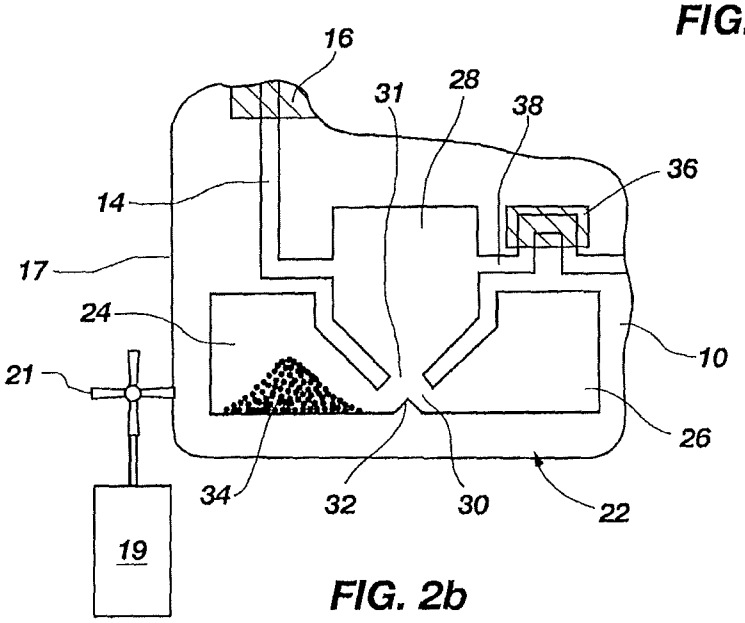

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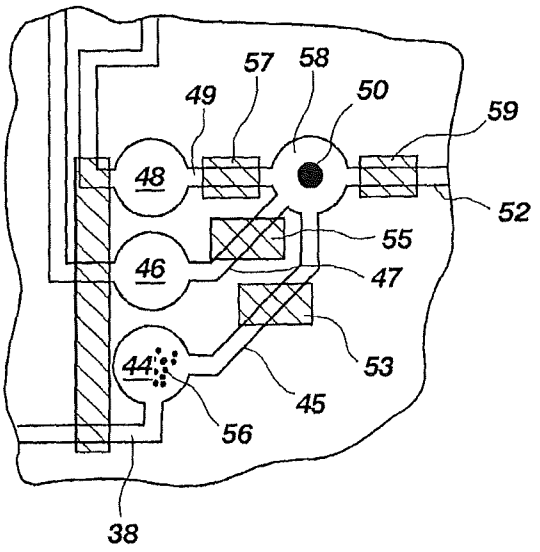
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FIG. 3

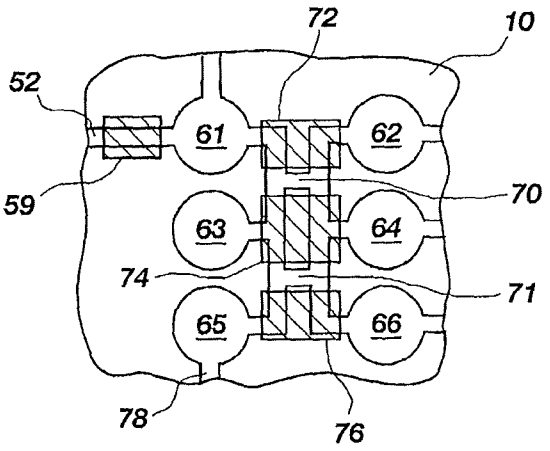
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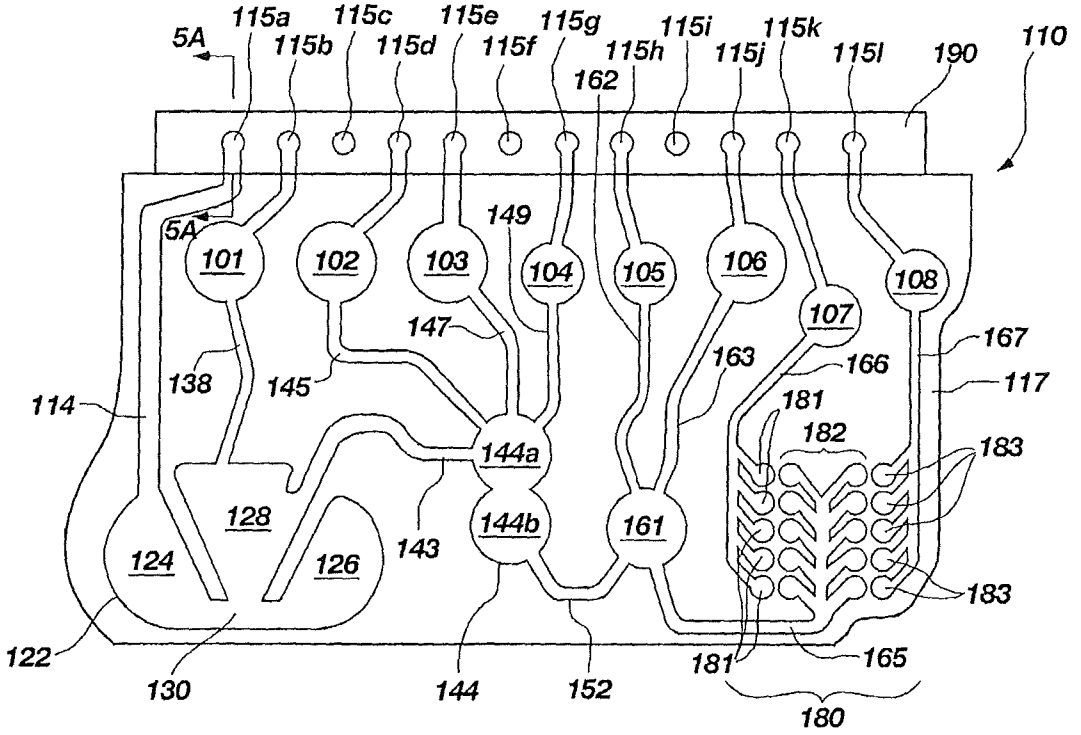
FIG. 4



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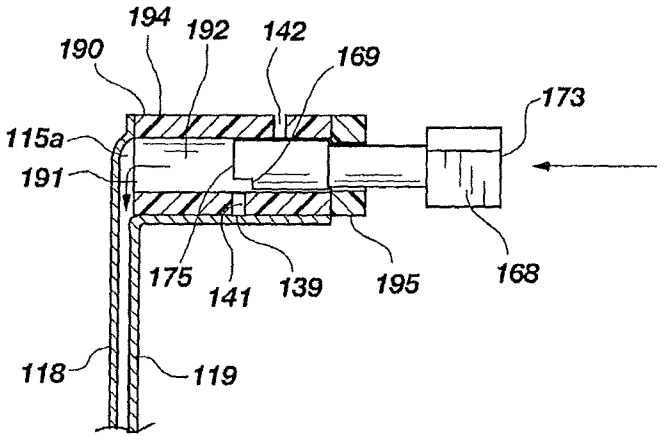
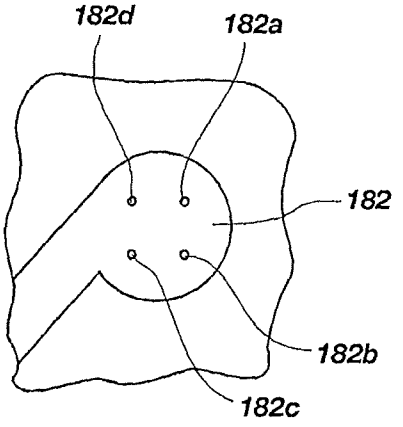


FIG. 5a



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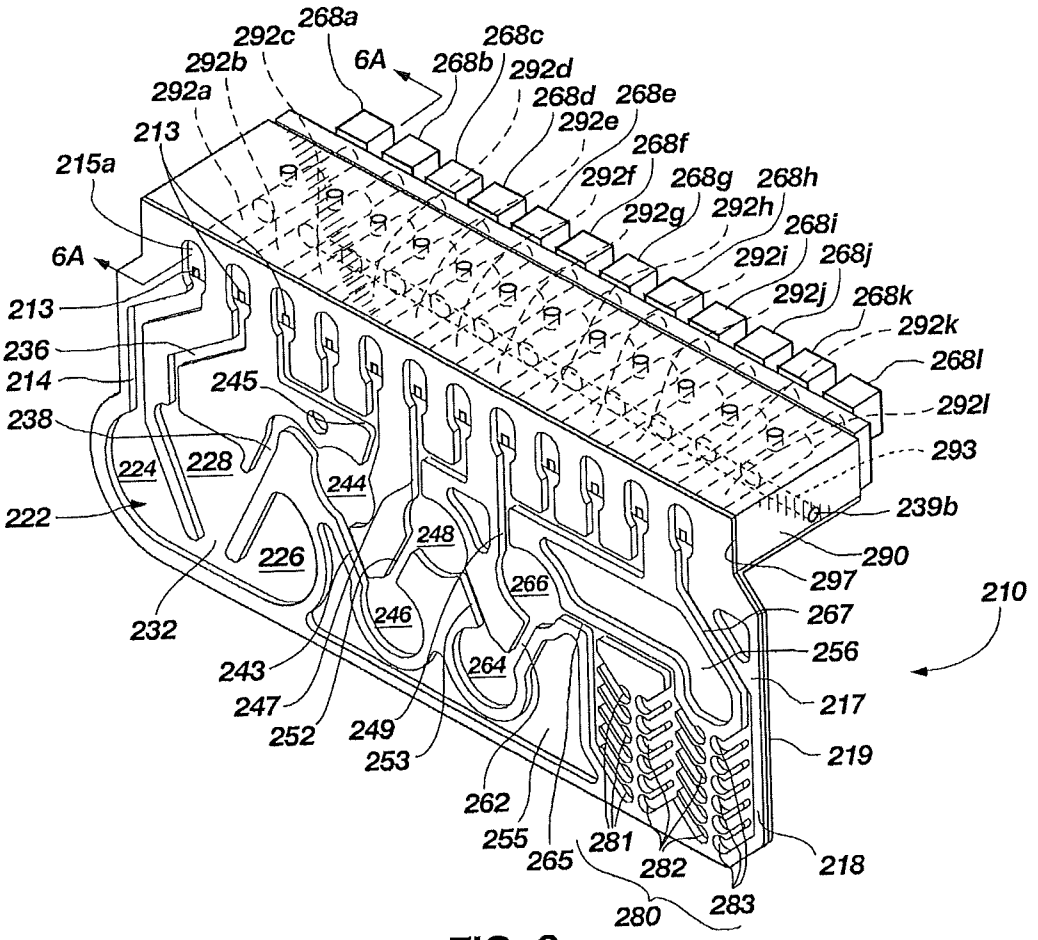
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268i
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FIG. 6

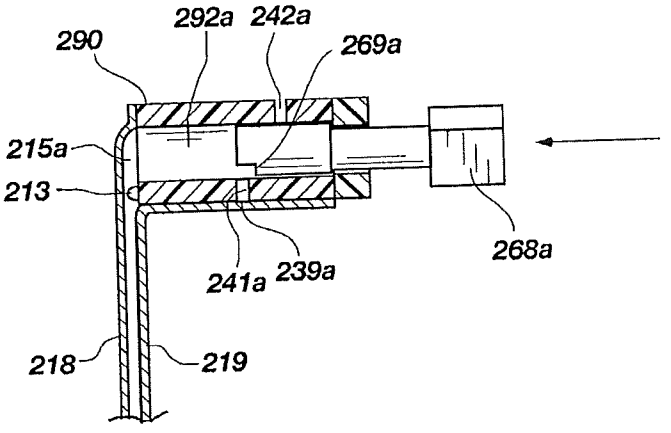
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FIG. 6a

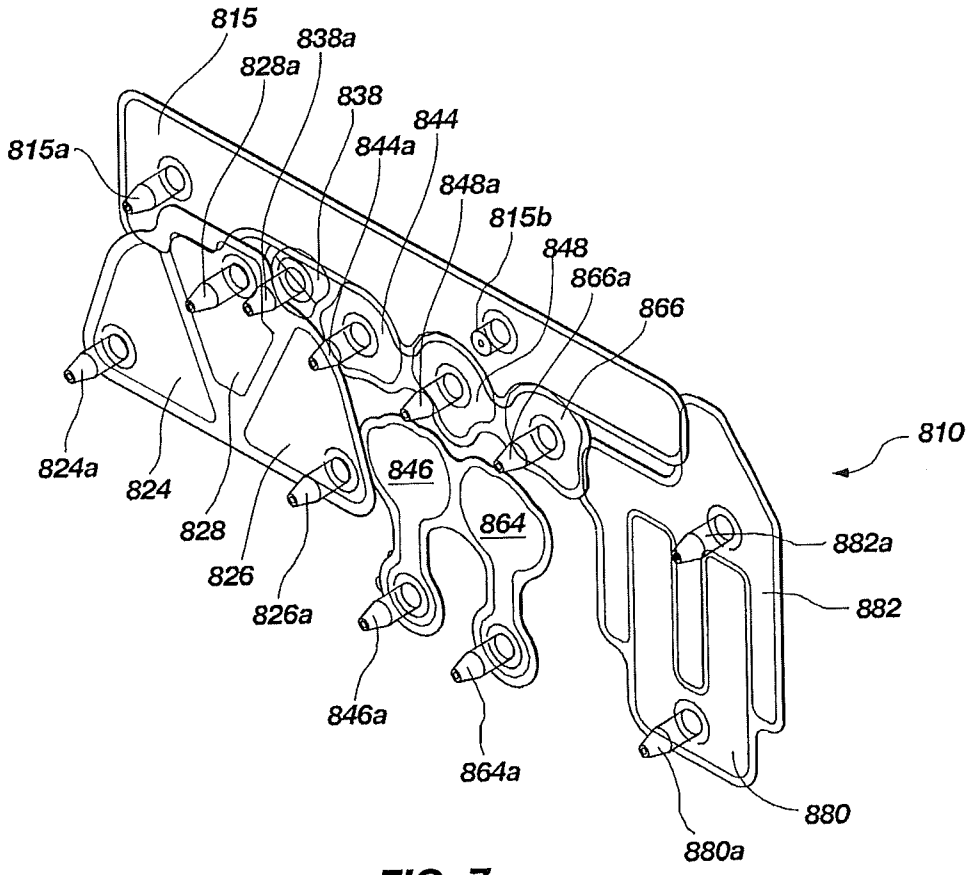
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FIG. 7

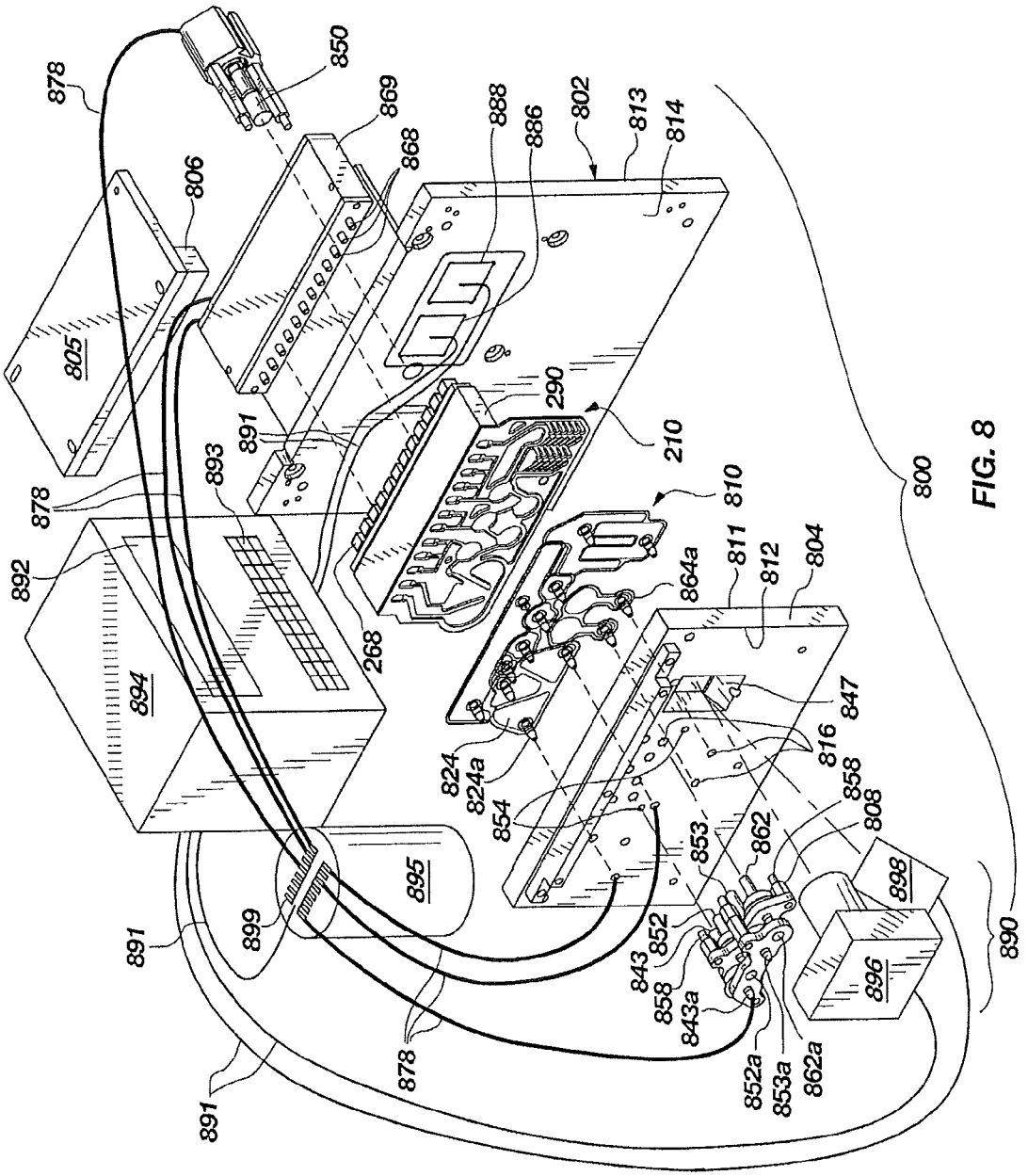
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FIG. 8

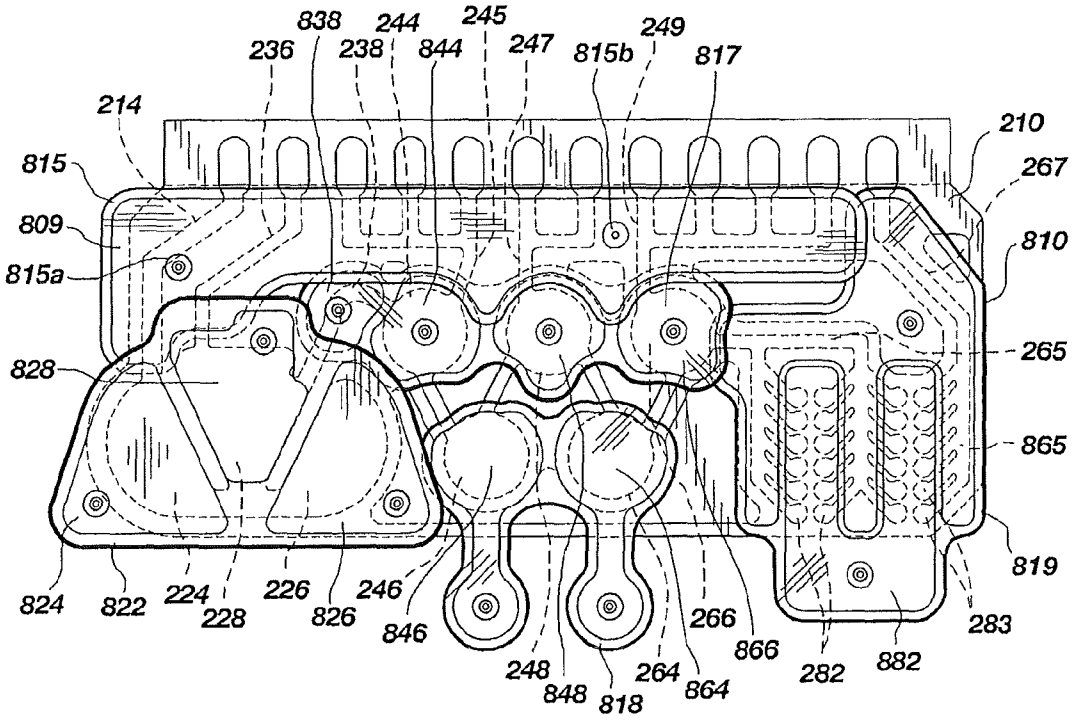
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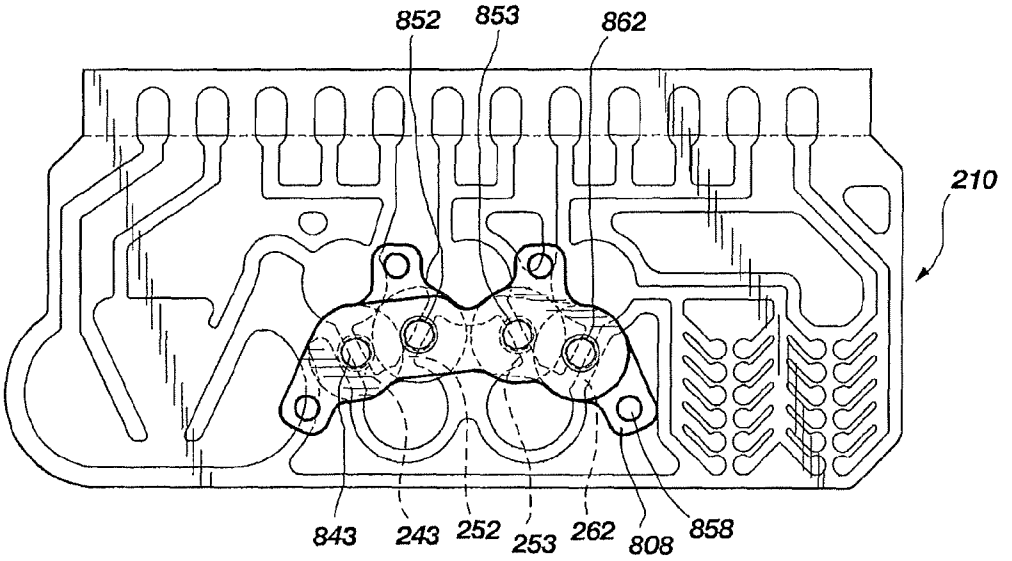
FIG. 9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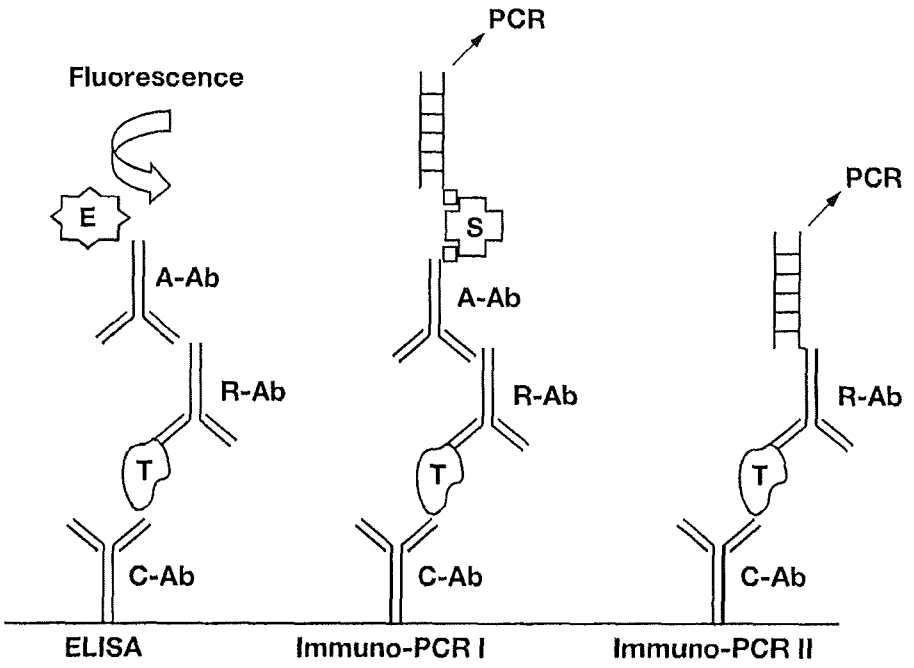
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Immuno-PCR I
Immuno-PCR II

FIG. 11

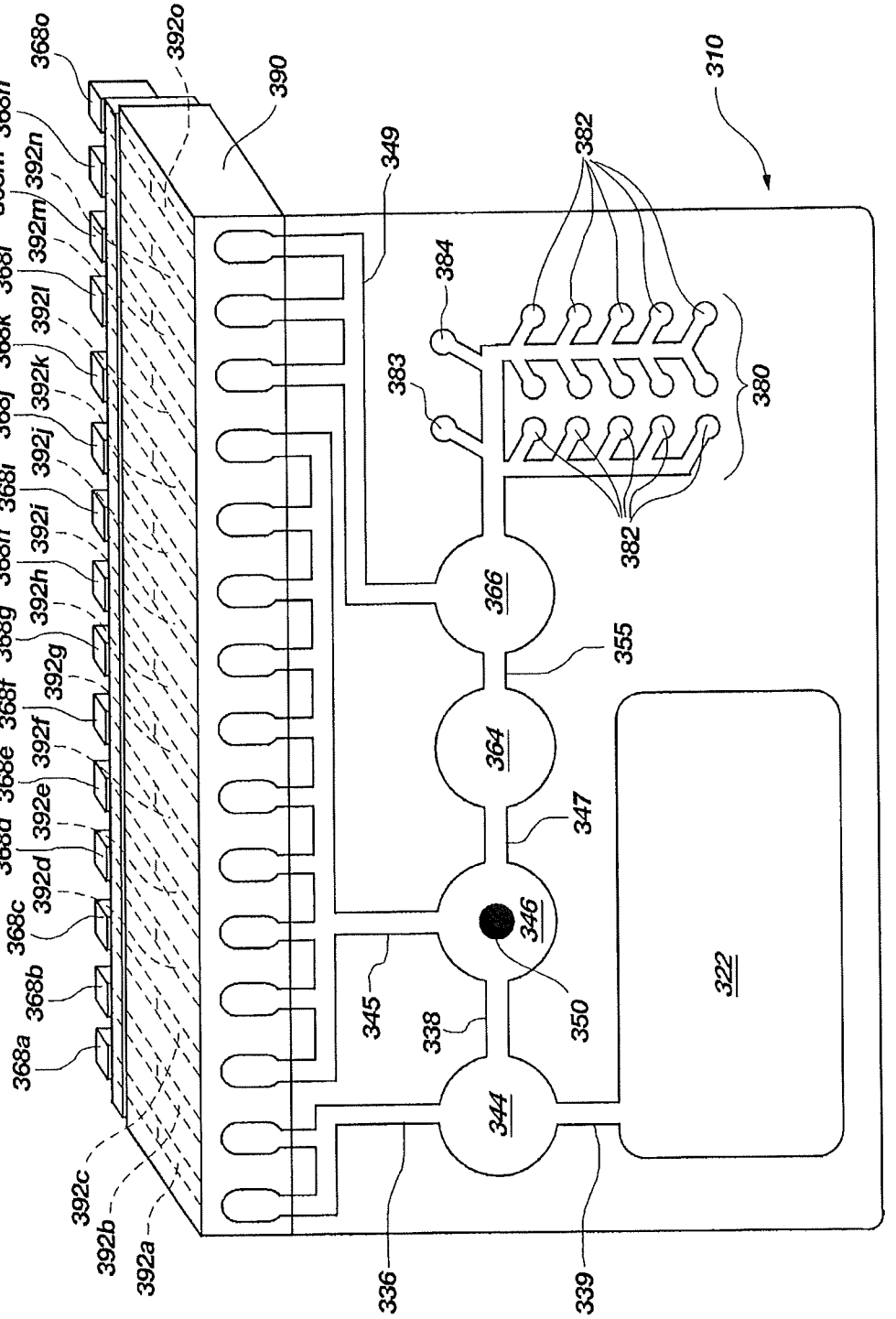
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FIG. 12

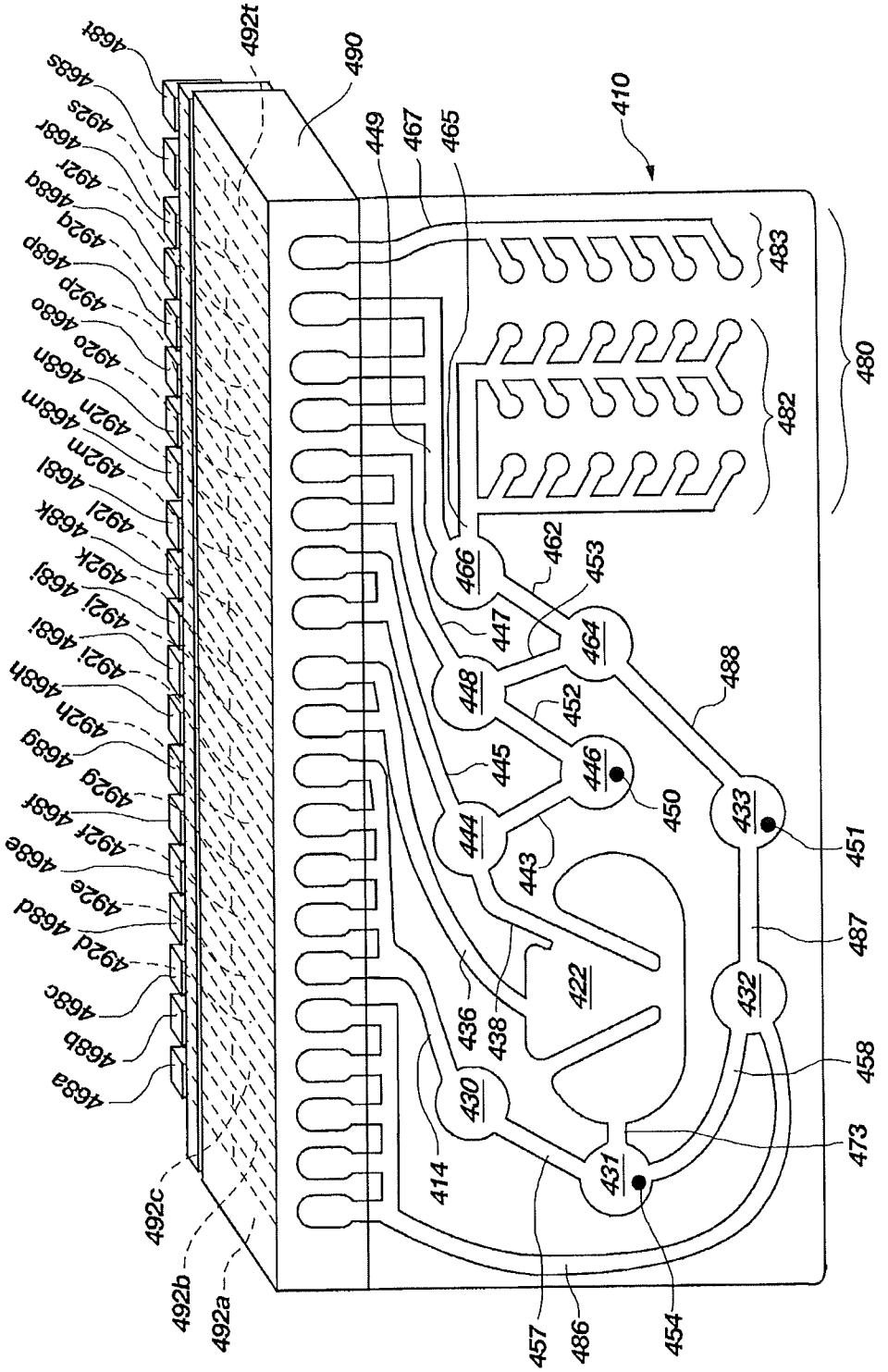
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FIG. 13

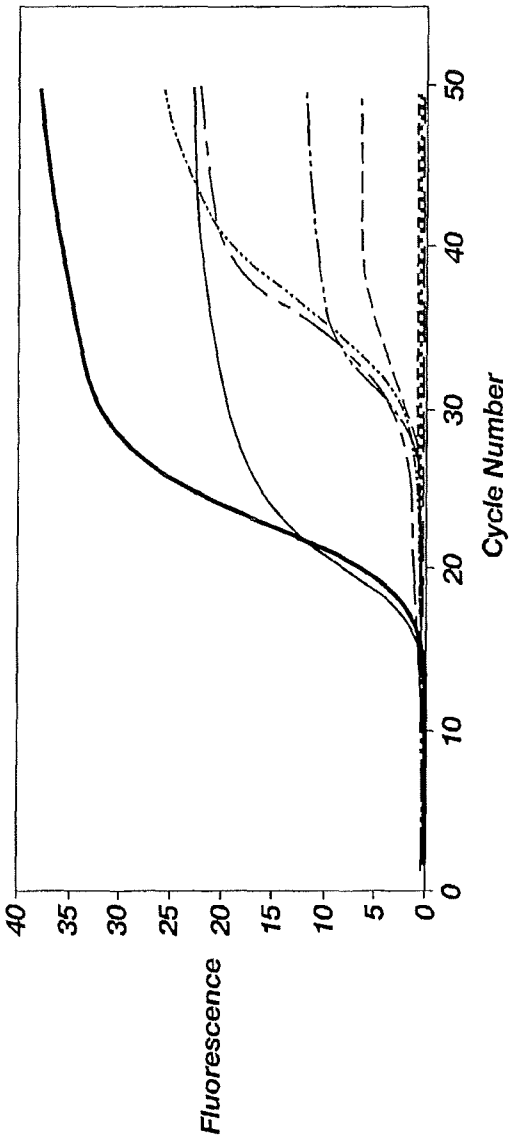
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FIG. 14

SELF-CONTAINED BIOLOGICAL ANALYSIS

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation of U.S. application Ser. No. 11/913,120, filed on Sep. 16, 2009, which is a national stage application of PCT Application Serial No. PCT/US2006/017665, filed on May 8, 2006; which claims priority from U.S. Application Ser. No. 60/679,052, filed on May 9, 2005, the entire disclosure of which is incorporated herein by reference.

GOVERNMENT INTEREST

This invention was made with government support under Grant Nos. U01 AI061611 and R43 AI063695 awarded by National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

In the United States, Canada, and Western Europe infectious disease accounts for approximately 7% of human mortality, while in developing regions infectious disease accounts for over 40% of human mortality. Infectious diseases lead to a variety of clinical manifestations. Among common overt manifestations are fever, pneumonia, meningitis, diarrhea, and diarrhea containing blood. While the physical manifestations suggest some pathogens and eliminate others as the etiological agent, a variety of potential causative agents remain, and clear diagnosis often requires a variety of assays be performed. Traditional microbiology techniques for diagnosing pathogens can take days or weeks, often delaying a proper course of treatment.

In recent years, the polymerase chain reaction (PCR) has become a method of choice for rapid diagnosis of infectious agents. PCR can be a rapid, sensitive, and specific tool to diagnose infectious disease. A challenge to using PCR as a primary means of diagnosis is the variety of possible causative organisms and the low levels of organism present in some pathological specimens. It is often impractical to run large panels of PCR assays, one for each possible causative organism, most of which are expected to be negative. The problem is exacerbated when pathogen nucleic acid is at low concentration and requires a large volume of sample to gather adequate reaction templates. In some cases there is inadequate sample to assay for all possible etiological agents. A solution is to run "multiplex PCR" wherein the sample is concurrently assayed for multiple targets in a single reaction. While multiplex PCR has proved to be valuable in some systems, shortcomings exist concerning robustness of high level multiplex reactions and difficulties for clear analysis of multiple products. To solve these problems, the assay may be subsequently divided into multiple secondary PCRs. Nesting secondary reactions within the primary product increases robustness. However, this further handling can be expensive and may lead to contamination or other problems.

Similarly, immuno-PCR ("iPCR") has the potential for sensitive detection of a wide variety of antigens. However, because traditional ELISA techniques have been applied to iPCR, iPCR often suffers from contamination issues that are problematic using a PCR-based detection method.

The present invention addresses various issues of contamination in biological analysis.

SUMMARY OF THE INVENTION

Accordingly, a rapid, sensitive assay that simultaneously assays for multiple biological substances, including organisms, is provided. The self-contained system illustratively employs an inexpensive disposable plastic pouch in a self-contained format, allowing for nested PCR and other means to identify bio-molecules, illustratively while minimizing contamination and providing for robust amplification.

Thus, in one aspect of the present invention a container for performing two-stage amplification on a sample in a closed system is provided, the container comprising a first-stage reaction zone comprising a first-stage reaction blister configured for first-stage amplification of the sample, an additional reservoir fluidly connected to the first-stage reaction blister, the additional reservoir configured for providing additional fluids to the sample, and a second-stage reaction zone fluidly connected to the first-stage reaction zone, the second-stage reaction zone comprising a plurality of second-stage reaction chambers, each second-stage reaction chamber comprising a pair of primers configured for further amplification of the sample. In one illustrative example, the first-stage reaction zone is a first-stage PCR amplification zone. In another illustrative example, the first stage reaction zone is an antigen-binding zone for immuno-PCR, in which antigens present in the sample are recognized and associated with a particular nucleic acid segment and the second stage reaction zone is a nucleic acid amplification zone. In yet another illustrative example, the container further comprises a cell lysis zone comprising particles for lysing cells or spores located in the sample, and a nucleic acid preparation zone comprising components for purifying nucleic acids. Illustratively, the blisters comprise a flexible material, such that pressure provided on an individual blister collapses the blister, forcing the contents from the blister.

In another aspect of the present invention, a container is provided comprising a flexible portion having a plurality of blisters fluidly connected via a plurality of channels, and a plurality of reservoirs, each reservoir containing a reaction component, and each reservoir fluidly connected to at least one of the plurality of blisters, and a sealable port configured for receiving the sample the sealable port fluidly connected to one of the plurality of blisters. In one illustrative embodiment, the reaction components are in dried form, and the container further comprises a second sealable port fluidly connected to each of the plurality of reservoirs, the port configured for receiving water to rehydrate the reaction components.

In a further aspect of the present invention, a method for lysing cells in a sample is provided, the method comprising providing a flexible container comprising a cell lysis blister, introducing cells into the cell lysis blister, and applying force to the blister to move the particles and sample to generate high velocity impacts resulting in a lysate.

In yet another aspect of the present invention, a device for analyzing a sample for the presence of nucleic acids is provided, the device configured to receive a container of the present invention therein, a plurality of actuators positioned corresponding to various blisters of the container, each actuator configured to apply pressure to the corresponding blister of the container, a first heater/cooler device configured for thermal cycling the contents of one of the blisters, and a second heater/cooler device for thermal cycling the second-stage chamber.

In still another aspect of the present invention, methods are provided. In one illustrative method, nucleic acids are amplified. In another illustrative method, antigens are detected using immuno-PCR.

Additional features of the present invention will become apparent to those skilled in the art upon consideration of the

following detailed description of preferred embodiments exemplifying the best mode of carrying out the invention as presently perceived.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows a flexible pouch according to one embodiment of this invention.

FIG. **2** shows an embodiment of the cell lysis zone of the flexible pouch according to FIG. **1**.

FIG. **2***a* shows an embodiment of a portion of a bladder corresponding to the cell lysis zone shown in FIG. **2**.

FIG. **2***b* shows an embodiment of the cell lysis zone of the flexible pouch according to FIG. **1** having an alternative vortexing mechanism.

FIG. **3** shows an embodiment of the nucleic acid preparation zone of the flexible pouch according to FIG. **1**.

FIG. **4** shows an embodiment of the first-stage amplification zone of the flexible pouch according to FIG. **1**.

FIG. **5** is similar to FIG. **1**, except showing an alternative embodiment of a pouch.

FIG. **5***a* is a cross-sectional view of the fitment of the pouch of FIG. **5**.

FIG. **5***b* is an enlargement of a portion of the pouch of FIG. **5**.

FIG. **6** is a perspective view of another alternative embodiment of a pouch.

FIG. **6***a* is a cross-sectional view of the fitment of the pouch of FIG. **6**.

FIG. **7** shows illustrative bladder components for use with the pouch of FIG. **6**.

FIG. **8** is an exploded perspective view of an instrument for use with the pouch of FIG. **6**, including the pouch of FIG. **6**.

FIG. **9** shows a partial cross-sectional view of the instrument of FIG. **8**, including the bladder components of FIG. **7**, with the pouch of FIG. **6** shown in shadow.

FIG. **10** shows a partial cross-sectional view of the instrument of FIG. **8**, including various bladders for pinch valves and the pouch of FIG. **6**.

FIG. **11** shows schemes for ELISA and immuno-PCR, secondary antibody (A); capture antibody (C); enzyme (E); reporter antibody (R); bi-functional binding moiety (S) and antigen (T).

FIG. **12** is similar to FIG. **6**, except showing a pouch configured for immuno-PCR. FIG. **13** is similar to FIG. **6**, except showing a pouch configured for both PCR and immuno-PCR.

FIG. **14** shows amplification curves from second-stage amplification of a sample that was lysed and amplified in a pouch of FIG. **5** (- - - positive control; — - — *S. cerevisaie* target **1**; ——— *S. cerevisaie* target **2**; ——— *S. cerevisaie* target **3**; — - - — - - *S. pombe* target **1**; — - - — *S. pombe* target **2**; - - - - negative controls).

DETAILED DESCRIPTION

The self-contained nucleic acid analysis pouches described herein may be used to assay a sample for the presence of various biological substances, illustratively antigens and nucleic acid sequences, illustratively in a single closed system. In one embodiment, the pouch is used to assay for multiple pathogens. Illustratively, various steps may be performed in the optionally disposable pouch, including nucleic acid preparation, primary large volume multiplex PCR, dilution of primary amplification product, and secondary PCR, culminating with real-time detection and/or post-amplification analysis such as melting-curve analysis. It is understood, however, that pathogen detection is one exemplary use and the pouches may be used for other nucleic acid analysis or detection of other substances, including but not limited to peptides, toxins, and small molecules. Further, it is understood that while the various steps may be performed in pouches of the present invention, one or more of the steps may be omitted for certain uses, and the pouch configuration may be altered accordingly.

FIG. **1** shows an illustrative self-contained nucleic acid analysis pouch **10**. Pouch **10** has a cell lysis zone **20**, a nucleic acid preparation zone **40**, a first-stage amplification zone **60**, and a second-stage amplification zone **80**. A sample containing nucleic acid is introduced into the pouch **10** via sample injection port **12**. Pouch **10** comprises a variety of channels and blisters of various sizes and is arranged such that the sample flows through the system. The sample passes through the various zones and is processed accordingly.

Sample processing occurs in various blisters located within pouch **10**. Various channels are provided to move the sample within and between processing zones, while other channels are provided to deliver fluids and reagents to the sample or to remove such fluids and reagents from the sample. Liquid within pouch **10** illustratively is moved between blisters by pressure, illustratively pneumatic pressure, as described below, although other methods of moving material within the pouch are contemplated.

While other containers may be used, illustratively, pouch **10** is formed of two layers of a flexible plastic film or other flexible material such as polyester, polyethylene terephthalate (PET), polycarbonate, polypropylene, polymethylmethacrylate, and mixtures thereof that can be made by any process known in the art, including extrusion, plasma deposition, and lamination. Metal foils or plastics with aluminum lamination also may be used. Other barrier materials are known in the art that can be sealed together to form the blisters and channels. If plastic film is used, the layers may be bonded together, illustratively by heat sealing. Illustratively, the material has low nucleic acid binding capacity.

For embodiments employing fluorescent monitoring, plastic films that are adequately low in absorbance and autofluorescence at the operative wavelengths are preferred. Such material could be identified by trying different plastics, different plasticizers, and composite ratios, as well as different thicknesses of the film. For plastics with aluminum or other foil lamination, the portion of the pouch that is to be read by a fluorescence detection device can be left without the foil. For example, if fluorescence is monitored in the blisters **82** of the second stage amplification zone **80** of pouch **10**, then one or both layers at blisters **82** would be left without the foil. In the example of PCR, film laminates composed of polyester (Mylar, Dupont, Wilmington Del.) of about 0.0048 inch (0.1219 mm) thick and polypropylene films of 0.001-0.003 inch (0.025-0.076 mm) thick perform well. Illustratively, pouch **10** is made of a clear material capable of transmitting approximately 80%-90% of incident light.

In the illustrative embodiment, the materials are moved between blisters by the application of pressure, illustratively pneumatic pressure, upon the blisters and channels. Accordingly, in embodiments employing pneumatic pressure, the pouch material illustratively is flexible enough to allow the pneumatic pressure to have the desired effect. The term "flexible" is herein used to describe a physical characteristic of the material of pouch. The term "flexible" is herein defined as readily deformable by the levels of pneumatic pressure used herein without cracking, breaking, crazing, or the like. For example, thin plastic sheets, such as Saran™ wrap and Ziploc® bags, as well as thin metal foil, such as aluminum

foil, are flexible. However, only certain regions of the blisters and channels need be flexible, even in embodiments employing pneumatic pressure. Further, only one side of the blisters and channels need to be flexible, as long as the blisters and channels are readily deformable. Other regions of the pouch **10** may be made of a rigid material or may be reinforced with a rigid material.

Illustratively, a plastic film is used for pouch **10**. A sheet of metal, illustratively aluminum, or other suitable material, may be milled or otherwise cut, to create a die having a pattern of raised surfaces. When fitted into a pneumatic press (illustratively A-5302-PDS, Janesville Tool Inc., Milton Wis.), illustratively regulated at an operating temperature of 195° C., the pneumatic press works like a printing press, melting the sealing surfaces of plastic film only where the die contacts the film. Various components, such as PCR primers (illustratively spotted onto the film and dried), antigen binding substrates, magnetic beads, and zirconium silicate beads may be sealed inside various blisters as the pouch **10** is formed. Reagents for sample processing can be spotted onto the film prior to sealing, either collectively or separately. In one embodiment, nucleotide tri-phosphates (NTPs) are spotted onto the film separately from polymerase and primers, essentially eliminating activity of the polymerase until the reaction is hydrated by an aqueous sample. If the aqueous sample has been heated prior to hydration, this creates the conditions for a true hot-start PCR and reduces or eliminates the need for expensive chemical hot-start components. This separate spotting is discussed further below, with respect to FIG. **5***b*, but it is understood that such spotting may be used with any of the embodiments discussed herein.

When pneumatic pressure is used to move materials within pouch **10**, in one embodiment a "bladder" may be employed. The bladder assembly **710**, a portion of which is shown in FIG. **2***a*, may be manufactured in a process similar to that of making the pouch, but individual blisters in the bladder assembly **710** include pneumatic fittings (illustratively fitting **724***a*) allowing individual bladders within the bladder assembly **710** to be pressurized by a compressed gas source. Because the bladder assembly is subjected to compressed gas and may be used multiple times, the bladder assembly may be made from tougher or thicker material than the pouch.

When pouch **10** is placed within the instrument, the pneumatic bladder assembly **710** is pressed against one face of the pouch **10**, so that if a particular bladder is inflated, the pressure will force the liquid out of the corresponding blister in the pouch **10**. In addition to pneumatic bladders corresponding to many of the blisters of pouch **10**, the bladder assembly may have additional pneumatic actuators, such as bladders or pneumatically-driven pistons, corresponding to various channels of pouch **10**. When activated, these additional pneumatic actuators form pinch valves to pinch off and close the corresponding channels. To confine liquid within a particular blister of pouch **10**, the pinch valve pneumatic actuators are inflated over the channels leading to and from the blister, such that the actuators function as pinch valves to pinch the channels shut. Illustratively, to mix two volumes of liquid in different blisters, the pinch valve pneumatic actuator sealing the connecting channel is depressurized, and the pneumatic bladders over the blisters are alternately pressurized, forcing the liquid back and forth through the channel connecting the blisters to mix the liquid therein. The pinch valve pneumatic actuators may be of various shapes and sizes and may be configured to pinch off more than one channel at a time. Such an illustrative pinch valve is illustrated in FIG. **1** as pinch valve **16**, which may be used to close all injection ports. While pneumatic actuators are discussed herein, it is understood that other ways of providing pressure to the pouch are contemplated, including various electromechanical actuators such as linear stepper motors, motor-driven cams, rigid paddles driven by pneumatic, hydraulic or electromagnetic forces, rollers, rocker-arms, and in some cases, cocked springs. In addition, there are a variety of methods of reversibly or irreversibly closing channels in addition to applying pressure normal to the axis of the channel. These include kinking the bag across the channel, heat-sealing, rolling an actuator, and a variety of physical valves sealed into the channel such as butterfly valves and ball valves. Additionally, small Peltier devices or other temperature regulators may be placed adjacent the channels and set at a temperature sufficient to freeze the fluid, effectively forming a seal. Also, while the design of FIG. **1** is adapted for an automated instrument featuring actuator elements positioned over each of the blisters and channels, it is also contemplated that the actuators could remain stationary, and the pouch could be transitioned in one or two dimensions such that a small number of actuators could be used for several of the processing stations including sample disruption, nucleic-acid capture, first and second-stage PCR, and other applications of the pouch such as immuno-assay and immuno-PCR. Rollers acting on channels and blisters could prove particularly useful in a configuration in which the pouch is translated between stations. Thus, while pneumatic actuators are used in the presently disclosed embodiments, when the term "pneumatic actuator" is used herein, it is understood that other actuators and other ways of providing pressure may be used, depending on the configuration of the pouch and the instrument.

With reference to FIG. **1**, an illustrative sample pouch **10** configured for nucleic acid extraction and multiplex PCR is provided. The sample enters pouch **10** via sample injection port **12** in fitment **90**. Injector port **12** may be a frangible seal, a one-way valve, or other entry port. Vacuum from inside pouch **10** may be used to draw the sample into pouch **10**, a syringe or other pressure may be used to force the sample into pouch **10**, or other means of introducing the sample into pouch **10** via injector port **12** may be used. The sample travels via channel **14** to the three-lobed blister **22** of the cell lysis zone **20**, wherein cells in the sample are lysed. Once the sample enters three-lobed blister **22**, pinch valve **16** is closed. Along with pinch valve **36**, which may have been already closed, the closure of pinch valve **16** seals the sample in three-lobed blister **22**. It is understood that cell lysis may not be necessary with every sample. For such samples, the cell lysis zone may be omitted or the sample may be moved directly to the next zone. However, with many samples, cell lysis is needed. In one embodiment, bead-milling is used to lyse the cells.

Bead-milling, by shaking or vortexing the sample in the presence of lysing particles such as zirconium silicate (ZS) beads **34**, is an effective method to form a lysate. It is understood that, as used herein, terms such as "lyse," "lysing," and "lysate" are not limited to rupturing cells, but that such terms include disruption of non-cellular particles, such as viruses. FIG. **2** displays one embodiment of a cell lysis zone **20**, where convergent flow creates high velocity bead impacts, to create lysate. Illustratively, the two lower lobes **24**, **26** of three-lobed blister **22** are connected via channel **30**, and the upper lobe **28** is connected to the lower lobes **24**, **26** at the opposing side **31** of channel **30**. FIG. **2***a* shows a counterpart portion of the bladder assembly **710** that would be in contact with the cell lysis zone **20** of the pouch **10**. When pouch **10** is placed in an instrument, adjacent each lobe **24**, **26**, **28** on pouch **10** is a corresponding pneumatic bladder **724**, **726**, **728** in the bladder assembly **710**. It is understood that the term "adjacent,"

when referring to the relationship between a blister or channel in a pouch and its corresponding pneumatic actuator, refers to the relationship between the blister or channel and the corresponding pneumatic actuator when the pouch is placed into the instrument. In one embodiment, the pneumatic fittings **724***a*, **726***a* of the two lower pneumatic bladders **724**, **726** adjacent lower lobes **24**, **26** are plumbed together. The pneumatic fittings **724***a*, **726***a* and the pneumatic fitting **728***a* of upper pneumatic bladder **728** adjacent upper lobe **28** are plumbed to the opposing side of an electrically actuated valve configured to drive a double-acting pneumatic cylinder. Thus configured, pressure is alternated between the upper pneumatic bladder **728** and the two lower pneumatic bladders **724**, **726**. When the valve is switched back and forth, liquid in pouch **10** is driven between the lower lobes **24**, **26** and the upper lobe **28** through a narrow nexus **32** in channel **30**. As the two lower lobes **24**, **26** are pressurized at the same time, the flow converges and shoots into the upper lobe **28**. Depending on the geometry of the lobes, the collision velocity of beads **34** at the nexus **32** may be at least about 12 m/sec, providing high-impact collisions resulting in lysis. The illustrative three-lobed system allows for good cell disruption and structural robustness, while minimizing size and pneumatic gas consumption. While ZS beads are used as the lysing particles, it is understood that this choice is illustrative only, and that other materials and particles of other shapes may be used. It is also understood that other configurations for cell lysis zone **20** are within the scope of this invention.

While a three-lobed blister is used for cell lysis, it is understood that other multi-lobed configurations are within the scope of this invention. For instance, a four-lobed blister, illustratively in a cloverleaf pattern, could be used, wherein the opposite blisters are pressurized at the same time, forcing the lysing particles toward each other, and then angling off to the other two lobes, which then may be pressurized together. Such a four-lobed blister would have the advantage of having high-velocity impacts in both directions. Further, it is contemplated that single-lobed blisters may be used, wherein the lysing particles are moved rapidly from one portion of the single-lobed blister to the other. For example, pneumatic actuators may be used to close off areas of the single-lobed blister, temporarily forming a multi-lobed blister in the remaining areas. It may also be possible to move the sample and lysing particles quickly enough to effect lysis within a single-lobed lysis blister without temporarily forming a multi-lobed blister. In an one such alternative embodiment, as shown in FIG. **2***b*, vortexing may be achieved by impacting the pouch with rotating blades or paddles **21** attached to an electric motor **19**. The blades **21** may impact the pouch at the lysis blister or may impact the pouch near the lysis blister, illustratively at an edge **17** adjacent the lysis blister. In such an embodiment, the lysis blister may comprise one or more blisters. Other actuation methods may also be used such as motor, pneumatic, hydraulic, or electromagnetically-driven paddles acting on the lobes of the device. Rollers or rotary paddles can be used to drive fluid together at the nexus **32** of FIG. **2**, illustratively if a recirculation means is provided between the upper and lower lobes and the actuator provides peristaltic pumping action. Other configurations are within the scope of this invention.

FIG. **2***a* also shows pneumatic bladder **716** with pneumatic fitting **716***a*, and pneumatic bladder **736** with pneumatic fitting **736***a*. When the pouch **10** is placed in contact with bladder assembly **710**, bladder **716** lines up with channel **12** to complete pinch valve **16**. Similarly, bladder **736** lines up with channel **38** to complete pinch valve **36**. Operation of pneumatic bladders **716** and **736** allow pinch valves **16** and **36** to be opened and closed. While only the portion of bladder assembly **710** adjacent the cell lysis zone is shown, it is understood that bladder assembly **710** would be provided with similar arrangements of pneumatic blisters to control the movement of fluids throughout the remaining zones of pouch **10**.

Other prior art instruments teach PCR within a sealed flexible container. See, e.g., U.S. Pat. Nos. 6,645,758 and 6,780,617, and co-pending U.S. patent application Ser. No. 10/478,453, herein incorporated by reference. However, including the cell lysis within the sealed PCR vessel can improve ease of use and safety, particularly if the sample to be tested may contain a biohazard. In the embodiments illustrated herein, the waste from cell lysis, as well as all other steps, remains within the sealed pouch. However, it is understood that the pouch contents could be removed for further testing.

Once the cells are lysed, pinch valve **36** is opened and the lysate is moved through channel **38** to the nucleic acid preparation zone **40**, as best seen in FIG. **3**, after which, pinch valve **36** is closed, sealing the sample in nucleic acid preparation zone **40**. In the embodiment illustrated in FIG. **3**, purification of nucleic acids takes the bead-milled material and uses affinity binding to silica-based magnetic-beads **56**, washing the beads with ethanol, and eluting the nucleic acids with water or other fluid, to purify the nucleic acid from the cell lysate. The individual components needed for nucleic acid extraction illustratively reside in blisters **44**, **46**, **48**, which are connected by channels **45**, **47**, **49** to allow reagent mixing. The lysate enters blister **44** from channel **38**. Blister **44** may be provided with magnetic beads **56** and a suitable binding buffer, illustratively a high-salt buffer such as that of 1-2-3 Sample Preparation Kit (Idaho Technology, Salt Lake City, Utah) or either or both of these components may be provided subsequently through one or more channels connected to blister **44**. The nucleic acids are captured on beads **56**, pinch valve **53** is then opened, and the lysate and beads **56** may be mixed by gentle pressure alternately on blisters **44** and **58** and then moved to blister **58** via pneumatic pressure illustratively provided by a corresponding pneumatic bladder on bladder assembly **710**. The magnetic beads **56** are captured in blister **58** by a retractable magnet **50**, which is located in the instrument adjacent blister **58**, and waste may be moved to a waste reservoir or may be returned to blister **44** by applying pressure to blister **58**. Pinch valve **53** is then closed. The magnetic beads **56** are washed with ethanol, isopropanol, or other organic or inorganic wash solution provided from blister **46**, upon release of pinch valve **55**. Optionally, magnet **50** may be retracted allowing the beads to be washed by providing alternate pressure on blisters **46** and **58**. The beads **56** are once again captured in blister **58** by magnet **50**, and the non-nucleic acid portion of the lysate is washed from the beads **56** and may be moved back to blister **46** and secured by pinch valve **55** or may be washed away via another channel to a waste reservoir. Once the magnetic beads are washed, pinch valve **57** is opened, releasing water (illustratively buffered water) or another nucleic acid eluant from blister **48**. Once again, the magnet **50** may be retracted to allow maximum mixing of water and beads **56**, illustratively by providing alternate pressure on blisters **48** and **58**. The magnet **50** is once again deployed to collect beads **56**. Pinch valve **59** is released and the eluted nucleic acid is moved via channel **52** to first-stage amplification zone **60**. Pinch valve **59** is then closed, thus securing the sample in first-stage amplification zone **60**.

It is understood that the configuration for the nucleic acid preparation zone **40**, as shown in FIG. **3** and described above,

is illustrative only, and that various other configurations are possible within the scope of the present disclosure.

The ethanol, water, and other fluids used herein may be provided to the blisters in various ways. The fluids may be stored in the blisters, the necks of which may be pinched off by various pinch valves or frangible portions that may be opened at the proper time in the sample preparation sequence. Alternatively, fluid may be stored in reservoirs in the pouch as shown pouch **110** in FIG. **5**, or in the fitment as discussed with respect to pouch **210** of FIG. **6**, and moved via channels, as necessary. In still another embodiment, the fluids may be introduced from an external source, as shown in FIG. **1**, especially with respect to ethanol injection ports **41**, **88** and plungers **67**, **68**, **69**. Illustratively, plungers **67**, **68**, **69** may inserted into fitment **90**, illustratively of a more rigid material, and may provide a measured volume of fluid upon activation of the plunger, as in U.S. patent application Ser. No. 10/512,255, herein incorporated by reference. The measured volume may be the same or different for each of the plungers. Finally, in yet another embodiment, the pouch may be provided with a measured volume of the fluid that is stored in one or more blisters, wherein the fluid is contained within the blister, illustratively provided in a small sealed pouch within the blister, effectively forming a blister within the blister. At the appropriate time, the sealed pouch may then be ruptured, illustratively by pneumatic pressure, thereby releasing the fluid into the blister of the pouch. The instrument may also be configured the provide some or all of the reagents directly through liquid contacts between the instrument and the fitment or pouch material provided that the passage of fluid is tightly regulated by a one-way valve to prevent the instrument from becoming contaminated during a run. Further, it will often be desirable for the pouch or its fitment to be sealed after operation to prohibit contaminating DNA to escape from the pouch. Various means are known to provide reagents on demand such as syringe pumps, and to make temporary fluid contact with the fitment or pouch, such as barbed fittings or o-ring seals. It is understood that any of these methods of introducing fluids to the appropriate blister may be used with any of the embodiments of the pouch as discussed herein, as may be dictated by the needs of a particular application.

As discussed above, nested PCR involves target amplification performed in two stages. In the first-stage, targets are amplified, illustratively from genomic or reverse-transcribed template. The first-stage amplification may be terminated prior to plateau phase, if desired. In the secondary reaction, the first-stage amplicons may be diluted and a secondary amplification uses the same primers or illustratively uses nested primers hybridizing internally to the primers of the first-stage product. Advantages of nested PCR include: a) the initial reaction product forms a homogeneous and specific template assuring high fidelity in the secondary reaction, wherein even a relatively low-efficiency first-stage reaction creates adequate template to support robust second-stage reaction; b) nonspecific products from the first-stage reaction do not significantly interfere with the second stage reaction, as different nested primers are used and the original amplification template (illustratively genomic DNA or reverse-transcription product) may be diluted to a degree that eliminates its significance in the secondary amplification; and c) nested PCR enables higher-order reaction multiplexing. First-stage reactions can include primers for several unique amplification products. These products are then identified in the second-stage reactions. However, it is understood that first-stage multiplex and second-stage singleplex is illustrative only and that other configurations are possible. For example, the first-stage may amplify a variety of different related amplicons using a single pair of primers, and second-stage may be used to target differences between the amplicons, illustratively using melting curve analysis.

Turning back to FIG. **1**, the nucleic acid sample enters the first-stage amplification zone **60** via channel **52** and is delivered to blister **61**. A PCR mixture, including a polymerase (illustratively a Taq polymerase), dNTPs, and primers, illustratively a plurality of pairs of primers for multiplex amplification, may be provided in blister **61** or may be introduced into blister **61** via various means, as discussed above. Alternatively, dried reagents may be spotted onto the location of blister **61** upon assembly of pouch **10**, and water or buffer may be introduced to blister **61**, illustratively via plunger **68**, as shown in FIG. **1**. As best seen in FIG. **4**, the sample is now secured in blister **61** by pinch valves **59** and **72**, and is thermocycled between two or more temperatures, illustratively by heat blocks or Peltier devices that are located in the instrument and configured to contact blister **61**. However, it is understood that other means of heating and cooling the sample contained within blister **61**, as are known in the art, are within the scope of this invention. Non-limiting examples of alternative heating/cooling devices for thermal cycling include having a air-cycled blister within the bladder, in which the air in the pneumatic blister adjacent blister **61** is cycled between two or more temperatures; or moving the sample to temperature zones within the blister **61**, illustratively using a plurality of pneumatic presses, as in U.S. patent application Ser. No. 10/478,453, herein incorporated by reference, or by translating pouch **10** on an axis or providing pouch **10** with a rotary layout and spinning pouch **10** to move the contents between heat zones of fixed temperature.

Nucleic acids from pathogens are often co-isolated with considerable quantities of host nucleic acids. These host-derived nucleic acids often interact with primers, resulting in amplification of undesired products that then scavenge primers, dNTPs, and polymerase activity, potentially starving a desired product of resources. Nucleic acids from pathogenic organisms are generally of low abundance, and undesired product is a potential problem. The number of cycles in the first-stage reaction of zone **60** may be optimized to maximize specific products and minimize non-specific products. It is expected that the optimum number of cycles will be between about 10 to about 30 cycles, illustratively between about 15 to about 20 cycles, but it is understood that the number of cycles may vary depending on the particular target, host, and primer sequence.

Following the first-stage multiplex amplification, the first-stage amplification product is diluted, illustratively in incomplete PCR master mix, before fluidic transfer to secondary reaction sites.

FIG. **4** shows an illustrative embodiment for diluting the sample in three steps. In the first step, pinch valve **72** is opened and the sample undergoes a two-fold dilution by mixing the sample in blister **61** with an equal volume of water or buffer from blister **62**, which is provided to blister **62**, as well as blisters **64** and **66**, as discussed above. Squeezing the volume back and forth between blisters **61**, **62** provides thorough mixing. As above, mixing may be provided by pneumatic bladders provided in the bladder **710** and located adjacent blisters **61**, **62**. The pneumatic bladders may be alternately pressurized, forcing the liquid back and forth. During mixing, a pinch valve **74** prevents the flow of liquid into the adjacent blisters. At the conclusion of mixing, a volume of the diluted sample is captured in region **70**, and pinch valve **72** is closed, sealing the diluted sample in region **70**. Pinch valve **74** is opened and the sample is further diluted by water or buffer provided in either or both of blisters **63**, **64**.

As above, squeezing the volume back and forth between blisters **63**, **64** provides mixing. Subsequently, pinch valve **74** is closed, sealing a further diluted volume of sample in region **71**. Final dilution takes place illustratively by using buffer or water provided in either or both of blisters **65**, **66**, with mixing as above. Illustratively this final dilution takes place using an incomplete PCR master mix (e.g., containing all PCR reagents except primers) as the fluid. Optional heating of the contents of blister **66** prior to second-stage amplification can provide the benefits of hot-start amplification without the need for expensive antibodies or enzymes. It is understood, however, that water or other buffer may be used for the final dilution, with additional PCR components provided in second-stage amplification zone **80**. While the illustrative embodiment uses three dilution stages, it is understood that any number of dilution stages may be used, to provide a suitable level of dilution. It is also understood that the amount of dilution can be controlled by adjusting the volume of the sample captured in regions **70** and **71**, wherein the smaller the amount of sample captured in regions **70** and **71**, the greater the amount of dilution or wherein additional aliquots captured in region **70** and/or region **71** by repeatedly opening and closing pinch valves **72** and **74** and/or pinch valves **74** and **76** may be used to decrease the amount of dilution. It is expected that about 10^{-2} to about 10^{-5} dilution would be suitable for many applications.

Success of the secondary PCR reactions is dependent upon template generated by the multiplex first-stage reaction. Typically, PCR is performed using DNA of high purity. Methods such as phenol extraction or commercial DNA extraction kits provide DNA of high purity. Samples processed through the pouch **10** may require accommodations be made to compensate for a less pure preparation. PCR may be inhibited by components of biological samples, which is a potential obstacle. Illustratively, hot-start PCR, higher concentration of taq polymerase enzyme, adjustments in $MgCl_2$ concentration, adjustments in primer concentration, and addition of adjuvants (such as DMSO, TMSO, or glycerol) optionally may be used to compensate for lower nucleic acid purity. While purity issues are likely to be more of a concern with first-stage amplification, it is understood that similar adjustments may be provided in the second-stage amplification as well.

Subsequent to first-stage PCR and dilution, channel **78** transfers the sample to a plurality of low volume blisters **82** for secondary nested PCR. In one illustrative embodiment, dried primers provided in the second-stage blisters are resuspended by the incoming aqueous material to complete the reaction mixture. Optionally, fluorescent dyes such as LCGreen® Plus (Idaho Technology, Salt Lake City, Utah) used for detection of double-stranded nucleic acid may be provided in each blister or may be added to the incomplete PCR master mix provided at the end of the serial dilution, although it is understood that LCGreen® Plus is illustrative only and that other dyes are available, as are known in the art. In another optional embodiment, dried fluorescently labeled oligonucleotide probes configured for each specific amplicon may be provided in each respective second-stage blister, along with the respective dried primers. Further, while pouch **10** is designed to contain all reactions and manipulations within, to reduce contamination, in some circumstances it may be desirable to remove the amplification products from each blister **82** to do further analysis. Other means for detection of the second-stage amplicon, as are known in the art, are within the scope of this invention. Once the sample is transferred to blisters **82**, pinch valves **84** and **86** are activated to close off blisters **82**. Each blister **82** now contains all reagents needed for amplification of a particular target. Illustratively, each blister may contain a unique pair of primers, or a plurality of blisters **82** may contain the same primers to provide a number of replicate amplifications.

It is noted that the embodiments disclosed herein use blisters for the second-stage amplification, wherein the blisters are formed of the same or similar plastic film as the rest of the flexible portion. However, in many embodiments, the contents of the second-stage blisters are never removed from the second-stage blisters, and, therefore, there is no need for the second-stage reaction to take place in flexible blisters. It is understood that the second-stage reaction may take place in a plurality of rigid, semi-rigid, or flexible chambers that are fluidly connected to the blisters. The chambers could be sealed as in the present example by placing pressure on flexible channels that connect the chambers, or may be sealed in other ways, illustratively by heat sealing or use of one-way valves. Various embodiments discussed herein include blisters provided solely for the collection of waste. Since the waste may never be removed, waste could be collected in rigid, semi-rigid, or flexible chambers.

It is within the scope of this invention to do the second-stage amplification with the same primers used in the first-stage amplification (see U.S. Pat. No. 6,605,451). However, it is often advantageous to have primers in second-stage reactions that are internal to the first-stage product such that there is no or minimal overlap between the first- and second-stage primer binding sites. Dilution of first-stage product largely eliminates contribution of the original template DNA and first-stage reagents to the second-stage reaction. Furthermore, illustratively, second-stage primers with a Tm higher than those used in the first-stage may be used to potentiate nested amplification. Illustratively, second-stage products may be between about 100 to about 140 base pairs and have Tm values of 65° C.+/−2° C. Tm of about 65° C. allows effective two-temperature amplification. In second-stage amplification, illustrative parameters of 94° C. for 0-5 seconds transitioning to 65° C. for 15 seconds are anticipated. Primer may be designed to avoid significant hairpins, hetero/homo-dimers and undesired hybridization. Because of the nested format, second-stage primers tolerate deleterious interactions far more so than primers used to amplify targets from genomic DNA in a single step. Optionally, hot-start is used on second-stage amplification.

If a fluorescent dye is included in second-stage amplification, illustratively as a dsDNA binding dye or as part of a fluorescent probe, as are known in the art, optics provided may be used to monitor amplification of one or more of the samples. Optionally, analysis of the shape of the amplification curve may be provided to call each sample positive or negative. Illustrative methods of calling the sample are discussed in U.S. Pat. No. 6,730,501, herein incorporated by reference. Alternatively, methods employing a crossing threshold may be used. A computer may be provided externally or within the instrument and may be configured to perform the methods and call the sample positive or negative based upon the presence or absence of second-stage amplification. It is understood, however, that other methods, as are known in the art, may be used to call each sample. Other analyses may be performed on the fluorescent information. One such non-limiting example is the use of melting curve analysis to show proper melting characteristics (e.g. Tm, melt profile shape) of the amplicon. The optics provided may be configured to capture images of all blisters **82** at once, or individual optics may be provided for each individual blister. Other configurations are within the scope of this invention.

FIG. **5** shows an alternative pouch **110**. In this embodiment, various reagents are loaded into pouch **110** via fitment **190**. FIG. **5***a* shows a cross-section of fitment **190** with one of a plurality of plungers **168**. It is understood that, while FIG. **5***a* shows a cross-section through entry channel **115***a*, as shown in the embodiment of FIG. **5**, there are 12 entry channels present (entry channel **115***a* through **115***l*), each of which may have its own plunger **168** for use in fitment **190**, although in this particular configuration, entry channels **115***c*, **115***f*, and **115***i* are not used. It is understood that a configuration having 12 entry channels is illustrative only, and that any number of entry channels and associated plungers may be used. In the illustrative embodiment, an optional vacuum port **142** of fitment **190** is formed through a first surface **194** of fitment **190** to communicate with chamber **192**. Optional vacuum port **142** may be provided for communication with a vacuum or vacuum chamber (not shown) to draw out the air from within pouch **110** to create a vacuum within chamber **192** and the various blisters and chambers of pouch **110**. Plunger **168** is then inserted far enough into chamber **192** to seal off vacuum port **142**. Chamber **192** is illustratively provided under a predetermined amount of vacuum to draw a desired volume of liquid into chamber **192** upon use. Additional information on preparing chamber **192** may be found in U.S. patent application Ser. No. 10/512,255, already incorporated by reference.

Illustrative fitment **190** further includes an injection port **141** formed in the second surface **195** of fitment **190**. Illustratively, injection port **141** is positioned closer to the plastic film portion of pouch **110** than vacuum port **142**, as shown in FIG. **5***a*, such that the plunger **168** is inserted far enough to seal off vacuum port **142**, while still allowing access to chamber **192** via injection port **141**. As shown, second surface **119** of plastic film portion **117** provides a penetrable seal **139** to prevent communication between chamber **192** and the surrounding atmosphere via injection port **141**. However, it is understood that second surface **119** optionally may not extend to injection port **141** and various other seals may be employed. Further, if another location for the seal is desired, for example on a first surface **194** of fitment **190**, injection port **141** may include a channel to that location on fitment **190**. U.S. patent application Ser. No. 10/512,255, already incorporated by reference, shows various configurations where the seal is located remotely from the injection port, and the seal is connected to the chamber via a channel. Also, U.S. patent application Ser. No. 10/512,255 discloses various configurations where channels connect a single seal to multiple chambers. Variations in seal location, as well as connection of a single injection port to multiple chambers, are within the scope of this invention. Optionally, seal **139** may be frangible and may be broken upon insertion of a cannula (not shown), to allow a fluid sample from within the cannula to be drawn into or forced into chamber **192**.

The illustrative plunger **168** of the pouch assembly **110** is cylindrical in shape and has a diameter of approximately 5 mm to be press-fit into chamber **192**. Plunger **168** includes a first end portion **173** and an opposite second end portion **175**. As shown in FIG. **5***a*, a notch **169** of plunger **168** is formed in second end portion **175**. In use, second end portion **175** is inserted part way into chamber **192**, and notch **169** may be aligned with injection port **141** to allow a fluid sample to be drawn into or injected into chamber **192**, even when plunger **168** is inserted far enough that plunger **168** would otherwise be blocking injection port **141**.

Illustratively, a fluid is placed in a container (not shown) with a syringe having a cannulated tip that can be inserted into injection port **141** to puncture seal **139** therein. In using an air-evacuated pouch assembly **110**, when seal **139** is punctured, the fluid is withdrawn from the container due to the negative pressure within chamber **192** relative to ambient air pressure. Fluid then passes through port **141** to fill chamber **192**. At this point, the fluid usually does not flow into the plastic film portion **117** of pouch **110**. Finally, the plunger **168** is inserted into chamber **192** such that second end portion **175** of plunger **168** approaches the bottom **191** of chamber **192**, to push a measured amount of the reagent or sample into the plastic film portion **117**. As shown, plunger **168** is configured such that upon full insertion, second end portion **175** does not quite meet bottom **191** of chamber **192**. The remaining space is useful in trapping bubbles, thereby reducing the number of bubbles entering plastic film portion **117**. However, in some embodiments it may be desirable for second end portion **175** to meet bottom **191** upon full insertion of plunger **168**. In the embodiment shown in FIG. **5**, entry channels **115***a*, **115***b*, **115***d*, **115***e*, **115***g*, **115***h*, **115***j*, **115***k*, and **115***l* all lead to reaction zones or reservoir blisters. It is understood that full insertion of the plunger associated with entry channel **115***a* would force a sample into three-lobed blister **122**, full insertion of the plunger associated with entry channel **115***b* would force a reagent into reservoir blister **101**, full insertion of the plunger associated with entry channel **115***d* would force a reagent into reservoir blister **102**, full insertion of the plunger associated with entry channel **115***e* would force a reagent into reservoir blister **103**, full insertion of the plunger associated with entry channel **115***g* would force a reagent into reservoir blister **104**, full insertion of the plunger associated with entry channel **115***h* would force a reagent into reservoir blister **105**, full insertion of the plunger associated with entry channel **115***j* would force a reagent into reservoir blister **106**, full insertion of the plunger associated with entry channel **115***k* would force a reagent into reservoir blister **107**, and full insertion of the plunger associated with entry channel **115***l* would force a reagent into reservoir blister **108**.

If a plunger design is used including notch **169** as illustrated in the embodiment shown in FIG. **5***a*, the plunger **168** may be rotated prior to being lowered, so as to offset notch **169** and to close off injection port **141** from communication with chamber **192**, to seal the contents therein. This acts to minimize any potential backflow of fluid through injection port **141** to the surrounding atmosphere, which is particularly useful when it is desired to delay in full insertion of the plunger. Although notch **169** is shown and described above with respect to plunger **168**, it is within the scope of this disclosure to close off injection port **141** soon after dispensing the fluid sample into the chamber **192** by other means, such as depressing plunger **168** toward the bottom of chamber **192**, heat sealing, unidirectional valves, or self-sealing ports, for example. If heat sealing is used as the sealing method, a seal bar could be included in the instrument such that all chambers are heat sealed upon insertion of the pouch into the instrument.

In the illustrative method, the user injects the sample into the injection port **141** associated with entry channel **115***a*, and water into the various other injection ports. The water rehydrates reagents that have been previously freeze-dried into chambers **192** associated with each of entry channels **115***b*, **115***d*, **115***e*, **115***g*, **115***h*, **115***j*, **115***k*, and **115***l*. The water may be injected through one single seal and then be distributed via a channel to each of the chambers, as shown in FIG. **6** below, or the water could be injected into each chamber independently. Alternatively, rather than injecting water to rehydrate dried reagents, wet reagents such as lysis reagents, nucleic acid extraction reagents, and PCR reagents may be injected into the appropriate chambers **192** of the fitment **190**.

Upon activation of the plunger **168** associated with entry channel **115***a*, the sample is forced directly into three-lobed blister **122** via channel **114**. The user also presses the remaining plungers **168**, forcing the contents out of each of the chambers **192** in fitment **190** and into reservoir blisters **101** through **108**. At this point, pouch **110** is loaded into an instrument for processing. While instrument **800**, shown in FIG. **8**, is configured for the pouch **210** of FIG. **6**, it is understood that modification of the configuration of the bladders of instrument **800** would render instrument **800** suitable for use with pouch **110**, or with pouches of other configurations.

In one illustrative example, upon depression of the plungers **168**, reservoir blister **101** now contains DNA-binding magnetic beads in isopropanol, reservoir blister **102** now contains a first wash solution, reservoir blister **103** now contains a second wash solution, reservoir blister **104** now contains a nucleic acid elution buffer, reservoir blister **105** now contains first-stage PCR reagents, including multiplexed first-stage primers, reservoir blister **106** now contains second-stage PCR reagents without primers, reservoir blister **107** now contains negative control PCR reagents without primers and without template, and reservoir blister **108** now contains positive control PCR reagents with template. However, it is understood that these reagents are illustrative only, and that other reagents may be used, depending upon the desired reactions and optimization conditions.

Once pouch **110** has been placed into instrument **800** and the sample has been moved to three-lobed blister **122**, the sample may be subjected to disruption by agitating the sample with lysing particles such as ZS or ceramic beads. The lysing particles may be provided in three-lobed blister **122**, or may be injected into three-lobed blister **122** along with the sample. The three-lobed blister **122** of FIG. **5** is operated in much the same way as three-lobed blister **22** of FIG. **1**, with the two lower lobes **124**, **126** pressurized together, and pressure is alternated between the upper lobe **128** and the two lower lobes **124**, **126**. However, as illustrated, lower lobes **124**, **126** are much more rounded than lower lobes **24**, **26**, allowing for a smooth flow of beads to channel **130** and allowing for high-speed collisions, even without the triangular flow separator at nexus **32**. As with three-lobed blister **22**, three-lobed blister **122** of FIG. **5** allows for effective lysis or disruption of microorganisms, cells, and viral particles in the sample. It has been found that a channel **130** having a width of about 3-4 mm, and illustratively about 3.5 mm, remains relatively clear of beads during lysis and is effective in providing for high-velocity collisions.

After lysis, nucleic-acid-binding magnetic beads are injected into upper lobe **128** via channel **138** by pressurizing a bladder positioned over reservoir blister **101**. The magnetic beads are mixed, illustratively more gently than with during lysis, with the contents of three-lobed blister **122**, and the solution is incubated, illustratively for about 1 minute, to allow nucleic acids to bind to the beads.

The solution is then pumped into the "figure 8" blister **144** via channel **143**, where the beads are captured by a retractable magnet housed in the instrument, which is illustratively pneumatically driven. The bead capture process begins by pressurizing all lobes **124**, **126**, and **128** of the bead milling apparatus **122**. This forces much of the liquid contents of **122** through channel **143** and into blister **144**. A magnet is brought into contact with the lower portion **144***b* of blister **144** and the sample is incubated for several seconds to allow the magnet to capture the beads from the solution, then the bladders adjacent to blister **122** are depressurized, the bladders adjacent blister portions **144***a* and **144***b* are pressurized, and the liquid is forced back into blister **122**. Since not all of the beads are captured in a single pass, this process may be repeated up to 10 times to capture substantially all of the beads in blister **144**. Then the liquid is forced out of blister **144**, leaving behind only the magnetic beads and the captured nucleic acids, and wash reagents are introduced into blister **144** in two successive washes (from reservoir blisters **102** and **103** via channels **145** and **147**, respectively). In each wash, the bladder positioned over the reservoir blister containing the wash reagent is pressurized, forcing the contents into blister **144**. The magnet is withdrawn and the pellet containing the magnetic beads is disrupted by alternatively pressurizing each of two bladders covering each lobe **144***a* and **144***b* of blister **144**. When the upper lobe **144***a* is compressed, the liquid contents are forced into the lower lobe **144***b*, and when the lower lobe **144***b* is compressed, the contents are forced into the upper lobe **144***a*. By agitating the solution in blister **144** between upper lobe **144***a* and lower lobe **144***b*, the magnetic beads are effectively washed of impurities. A balance is maintained between inadequate agitation, leaving the pellet of beads undisturbed, and excessive agitation, potentially washing the nucleic acids from the surface of the beads and losing them with the wash reagents. After each wash cycle, the magnetic beads are captured via the magnet in blister **144** and the wash reagents are illustratively forced into three-lobed blister **122**, which now serves as a waste receptacle. However, it is understood that the used reservoir blisters may also serve as waste receptacles, or other blisters may be provided specifically as waste receptacles.

Nucleic acid elution buffer from reservoir blister **104** is then injected via channel **149** into blister **144**, the sample is once again agitated, and the magnetic beads are recaptured by employment of the magnet. The fluid mixture in blister **144** now contains nucleic acids from the original sample. Pressure on blister **144** moves the nucleic acid sample to the first stage PCR blister **161** via channel **152**, where the sample is mixed with first-stage PCR master mix containing multiple primer sets, the PCR master mix provided from reservoir blister **105** via channel **162**. If desired, the sample and/or the first-stage PCR master mix may be heated prior to mixing, to provide advantages of hot start. As will be seen below, pouch **110** of FIG. **5** is configured for up to 10 primer sets, but it is understood that the configuration may be altered and any number of primer sets may be used. A bladder positioned over blister **161** is pressurized at low pressure, to force the contents of blister **161** into intimate contact with a heating/cooling element, illustratively a Peltier element, on the other side of blister **161**. The pressure on blister **161** should be sufficient to assure good contact with the heating/cooling element, but should be gentle enough such that fluid is not forced from blister **161**. The heating/cooling element is temperature cycled, illustratively between about 60° C. to about 95° C. Illustratively, temperature cycling is performed for about 15-20 cycles, resulting in amplification of one or more nucleic acid targets present. Also illustratively, temperature cycling ceases prior to plateau phase, and may cease in log phase or even prior to log phase. In one example, it may be desirable merely to enrich the sample with the desired amplicons, without reaching minimal levels of detection. See U.S. Pat. No. 6,605,451, herein incorporated by reference.

The amplified sample is optionally then diluted by forcing most the sample back into blister **144** via channel **152**, leaving only a small amount (illustratively about 1 to 5%) of the amplified sample in blister **161**, and second-stage PCR master mix is provided from reservoir blister **106** via channel **163**. The sample is thoroughly mixed illustratively by moving it back and forth between blisters **106** and **161** via channel **163**. If desired, the reaction mixture may be heated prior to second-

stage amplification. The sample is then forced through channel **165** into an array of low volume blisters **182** in the center of second-stage amplification zone **180**. Each of the ten illustrative low volume blisters **182** may contain a different primer pair, either essentially the same as one of the primer pairs in the first-stage amplification, or "nested" within the first-stage primer pair to amplify a shortened amplicon. The primers, now hydrated by the sample, complete the amplification mixture. Positive and negative control samples are also introduced by pressurizing the contents of reservoir blisters **107** and **108**, respectively, forcing PCR master mix either without target DNA from reservoir blister **107** via channel **166**, or with control DNA from reservoir blister **108**, via channel **167**. As illustrated, there are five each of positive control blisters **183** and negative control blisters **181**, which may be multiplexed 2-fold to provide the necessary controls for ten different second-stage amplification reactions. It is understood that this configuration is illustrative only and that any number of second-stage blisters may be provided.

Illustratively, the PCR master mix used for second-stage amplification lacks the primers, but is otherwise complete. However, an "incomplete" PCR master mix may lack other PCR components as well. In one example, the second-stage PCR master mix is water or buffer only, which is then mixed with the optionally diluted first-stage PCR amplification product. This mixture is moved to the small-volume PCR reaction blisters, where all of the remaining components have been previously provided. If desired, all of the remaining components may be mixed together and spotted as a single mixture into the small-volume PCR reaction blisters. Alternatively, as illustrated in FIG. **5***b*, each of the components may be spotted onto a separate region of the small-volume PCR reaction blister **182**. As shown in FIG. **5***b*, four regions are present, illustratively with dNTPs spotted at region **182***a*, primers spotted at, polymerase spotted at **182***c*, and a magnesium compound spotted at **182***d*. By spotting the components separately and heating the sample mixture prior to rehydrating the components, nonspecific reactions can be minimized. It is understood that any combination of components can be spotted this way, and that this method of spotting components into one or more regions of the blisters may be used with any embodiment of the present invention.

The channels **165**, **166**, and **167** leading to the small-volume PCR reaction blisters **181**, **182**, and **183** are sealed, and a pneumatic bladder gently presses the array against a heating/cooling element, illustratively a Peltier element, for thermal cycling. The cycling parameters may be independently set for second-stage thermal cycling. Illustratively, the reactions are monitored by focusing an excitation source, illustratively a blue light (450-490 nm), onto the array, and imaging the resultant fluorescent emissions, illustratively fluorescent emissions above 510 nm.

In the above example, pinch valves are not discussed. However, it is understood that when it is desired to contain a sample in one of the blisters, pneumatic actuators positioned over channels leading to and from the particular blister are pressurized, creating pinch valves and closing off the channels. Conversely, when it is desired to move a sample from one of the blisters, the appropriate pneumatic actuator is depressurized, allowing the sample flow through the channel.

The pouch described above in FIG. **5** includes reagent reservoir blisters **101** through **108**, in which the user injected reagents from the fitment **190** into the reagent reservoir blisters **101** through **108** in the plastic film portion **117** of the pouch **110**, illustratively prior to insertion of pouch **110** into the instrument. While there are advantages to the use of the reagent reservoir blisters of FIG. **5**, including having the ability to maintain the contents of the various blisters at different temperatures, there are some disadvantages as well. Because the operator is responsible for moving the reagents from the fitment **190** to the reservoir blisters **101** through **108**, and because this is often done outside of the machine and thus without activated pinch valves, reagents could occasionally leak from the reservoir blisters to the working blisters. The reagents in reservoir blisters are exposed during preparation and loading. If they are pressed, squeezed, or even lightly bumped, the reagents may leak through available channels. If the loss of reagents is substantial, the reaction may fail completely. Furthermore, during operation there may be some variability in the amount of reagent forced from the reservoir blisters **101** through **108**, leading to inconsistent results. Automation of introduction of the reagents to fitment **190** and movement of the reagents from fitment **190** to reagent reservoir blisters **101** through **108** would solve many of these problems, and is within the scope of this invention.

The pouch **210** of FIG. **6** addresses many of these issues in a different way, by using a direct-injection approach wherein the instrument itself moves the plungers **268**, illustratively via pneumatic pistons, and forces the reagents into the various working blisters as the reagents are needed. Rather than storing the reagents in reservoir blisters **101** through **108** of FIG. **5**, in the embodiment of FIG. **6** the reagents are introduced into various chambers **292** of fitment **290** and are maintained there until needed. Pneumatic operation of piston **268** at the appropriate time introduces a measured amount of the reagent to the appropriate reaction blister. In addition to addressing many of the above-mentioned issues, pouch **210** also has a much more compact shape, allowing for a smaller instrument design, and pouch **210** has shorter channels, permitting better fluid flow and minimizing reagent loss in channels.

In one illustrative embodiment of FIG. **6**, a 300 μl mixture comprising the sample to be tested (100 μl) and lysis buffer (200 μl) is injected into injection port **241***a*. Water is also injected into the fitment **290** via seal **239***b*, hydrating up to eleven different reagents, each of which were previously provided in dry form in chambers **292***b* through **292***l* via channel **293** (shown in shadow). These reagents illustratively may include freeze-dried PCR reagents, DNA extraction reagents, wash solutions, immunoassay reagents, or other chemical entities. For the example of FIG. **6**, the reagents are for nucleic acid extraction, first-stage multiplex PCR, dilution of the multiplex reaction, and preparation of second-stage PCR reagents, and control reactions. In the embodiment shown in FIG. **6**, all that need be injected is the sample in port **241***a* and water in port **241***b*.

As shown in FIG. **6**, water injected via seal **293***b* is distributed to various chambers via channel **293**. In this embodiment, only the sample and water need be injected into pouch **210**. It is understood, however, that water could be injected into each chamber **292** independently. Further, it is understood that, rather than providing dried reagents in the various chambers **292** and hydrating upon injection of the water, specific wet reagents could be injected into each chamber, as desired. Additionally, it is understood that one or more of chambers **292** could be provided with water only, and the necessary reagents may be provided dried in the appropriate reaction blisters. Various combinations of the above, as dictated by the needs of the particular reaction, are within the scope of this invention.

As seen in FIG. **6**, optional protrusions **213** are provided on bottom surface **297** of fitment **290**. As shown, protrusions **213** are located within their respective entry channels **215**. However, other configurations are possible. Protrusions **213** assist in opening entry channel **215** and prevent bottom surface **297**

from engaging another flat surface in such a way to pinch off entry channels **215** when plungers **268** are depressed, which helps prevent back-flow upon activation of the plungers **268**. Such protrusions may be used on any of the various pouches according to the present invention.

In embodiments wherein water is injected into the pouch to hydrate multiple dry reagents in multiple chambers in the fitment, a means of closing the channel between the injection port and the many chambers is desired. If the channel is not closed, activation of each plunger may force some of the contents of its respective chamber back out into the channel, potentially contaminating neighboring chambers and altering the volumes contained in and delivered from the chamber. Several ways of closing this channel have been used, including rotating a notched plunger **268** as discussed above, and heat-sealing the plastic film across the channel thereby closing the channel permanently, and applying pressure to the channel as a pinch valve. Other closures may also be used, such as valves built into the fitment, illustratively one-way valves.

After the fluids are loaded into chambers **292** and pouch **210** is loaded into the instrument, plunger **268***a* is depressed illustratively via activation of a pneumatic piston, forcing the balance of the sample into three-lobed blister **220** via channel **214**. As with the embodiments shown in FIGS. **1** and **5**, the lobes **224**, **226**, and **228** of three-lobed blister **220** are sequentially compressed via action bladders **824**, **826**, and **828** of bladder assembly **810**, shown in FIGS. **7-9**, forcing the liquid through the narrow nexus **232** between the lobes, and driving high velocity collisions, shearing the sample and liberating nucleic acids, illustratively including nucleic acids from hard-to-open spores, bacteria, and fungi. Cell lysis continues for an appropriate length of time, illustratively 0.5 to 10 minutes.

Once the cells have been adequately lysed, plunger **268***b* is activated and nucleic acid binding magnetic beads stored in chamber **292***b* are injected via channel **236** into upper lobe **228** of three-lobed blister **220**. The sample is mixed with the magnetic beads and the mixture is allowed to incubate for an appropriate length of time, illustratively approximately 10 seconds to 10 minutes.

The mixture of sample and beads are forced through channel **238** into blister **244** via action of bladder **826**, then through channel **243** and into blister **246** via action of bladder **844**, where a retractable magnet **850** located in instrument **800** adjacent blister **245**, shown in FIG. **8**, captures the magnetic beads from the solution, forming a pellet against the interior surface of blister **246**. A pneumatic bladder **846**, positioned over blister **246** then forces the liquid out of blister **246** and back through blister **244** and into blister **222**, which is now used as a waste receptacle. However, as discussed above with respect to FIG. **5**, other waste receptacles are within the scope of this invention. One of plungers **268***c*, **268***d*, and **268***e* may be activated to provide a wash solution to blister **244** via channel **245**, and then to blister **246** via channel **243**. Optionally, the magnet **850** is retracted and the magnetic beads are washed by moving the beads back and forth from blisters **244** and **246** via channel **243**, by alternatively pressurizing bladders **844** and **846**. Once the magnetic beads are washed, the magnetic beads are recaptured in blister **246** by activation of magnet **850**, and the wash solution is then moved to blister **222**. This process may be repeated as necessary to wash the lysis buffer and sample debris from the nucleic acid-binding magnetic beads. Illustratively, three washes are done, one each using wash reagents in chambers **292***c*, **292***d*, and **292***e*. However, it is understood that more or fewer washes are within the scope of this invention. If more washes are desired, more chambers **292** may be provided. Alternatively, each chamber **292** may hold a larger volume of fluid and activation of the plungers may force only a fraction of the volume from the chamber upon each activation.

After washing, elution buffer stored in chamber **292***f* is moved via channel **247** to blister **248**, and the magnet is retracted. The solution is cycled between blisters **246** and **248** via channel **252**, breaking up the pellet of magnetic beads in blister **246** and allowing the captured nucleic acids to dissociate from the beads and come into solution. The magnet **850** is once again activated, capturing the magnetic beads in blister **246**, and the eluted nucleic acid solution is forced into blister **248**.

Plunger **268***h* is depressed and first-stage PCR master mix from chamber **292***h* is mixed with the nucleic acid sample in blister **248**. Optionally, the mixture is mixed by alternative activation of bladders **848** and **864**, forcing the mixture between **248** and **264** via channel **253**. After several cycles of mixing, the solution is contained in blister **264**, where first-stage multiplex PCR is performed. If desired, prior to mixing, the sample may be retained in blister **246** while the first-stage PCR master mix is pre-heated, illustratively by moving the first-stage PCR master mix into blister **264** or by providing a heater adjacent blister **248**. As discussed above, this pre-heating may provide the benefits of hot start PCR. The instrument **800** illustrated in FIG. **8** features Peltier-based thermal cyclers **886** and **888** which heat and cool the sample. However, it is understood that other heater/cooler devices may be used, as discussed above. Temperature cycling is illustratively performed for 15-20 cycles, although other levels of amplification may be desirable, depending on the application, as discussed above. As will be seen below, the second-stage amplification zone **280** is configured to detect amplification in 18 second-stage reactions. Accordingly, 18 different primer-pairs may be included in the PCR reaction in blister **264**.

After first-stage PCR has proceeded for the desired number of cycles, the sample may be diluted as discussed above with respect to the embodiment of FIG. **5**, by forcing most of the sample back into blister **248**, leaving only a small amount, and adding second-stage PCR master mix from chamber **292***i*. Alternatively, a dilution buffer from **292***i* may be moved to blister **266** via channel **249** and then mixed with the amplified sample in blister **264** by moving the fluids back and forth between blisters **264** and **266**. After mixing, a portion of the diluted sample remaining in blister **264** is forced away to three-lobed blister **222**, now the waste receptacle. If desired, dilution may be repeated several times, using dilution buffer from chambers **292***j* and **292***k*, and then adding second-stage PCR master mix from chamber **292***g* to some or all of the diluted amplified sample. It is understood that the level of dilution may be adjusted by altering the number of dilution steps or by altering the percentage of the sample discarded prior to mixing with the dilution buffer or second-stage PCR master mix. If desired, this mixture of the sample and second-stage PCR master mix may be pre-heated in blister **264** prior to movement to second-stage blisters **282** for second-stage amplification.

The illustrative second-stage PCR master mix is incomplete, lacking primer pairs, and each of the 18 second-stage blisters **282** is pre-loaded with a specific PCR primer pair. If desired, second-stage PCR master mix may lack other reaction components, and these components may then be pre-loaded in the second-stage blisters **282** as well. As discussed above with the prior examples, each primer pair may be identical to a first-stage PCR primer pair or may be nested within the first-stage primer pair. Movement of the sample

from blister **264** to the second-stage blisters completes the PCR reaction mixture. Control samples from chamber **292***l* are also moved to control blisters **283** via channel **267**. The control samples may be positive or negative controls, as desired. Illustratively, each pouch would contain control reactions that validate the operation of each step in the process and demonstrate that positive results are not the result of self-contamination with previously amplified nucleic acids. However, this is not practical in many protocols, particularly for a highly multiplexed reaction. One illustrative way of providing suitable controls involves spiking samples with a species such as baker's yeast. The nucleic acids are extracted from the yeast, alongside other nucleic acids. First- and second-stage PCR reactions amplify DNA and/or RNA targets from the yeast genome. Illustratively, an mRNA sequence derived from a spliced pre-mRNA can be used to generate an RNA-specific target sequence by arranging the primer sequences to span an intron. A quantative analysis of the yeast copy number against reference standards allows substantial validation that each component of the system is working. Negative control reactions for each of the many second-stage assays are more problematic. It may be desirable to run control reactions either in parallel or in a separate run.

Activation of bladder **882** of bladder assembly **810** seals the samples into their respective second-stage blisters **282**, **283**, and activation of bladder **880** provides gentle pressure on second-stage blisters **282**, **283**, to force second-stage blisters **282**, **283** into contact with a heater/cooler device. A window **897** positioned over the second-stage amplification zone **280** allows fluorescence monitoring of the array during PCR and during a DNA melting-curve analysis of the reaction products.

It is noted that the pouch **210** of FIG. **6** has several unsealed areas, such as unsealed area **255** and unsealed area **256**. These unsealed areas form blisters that are not involved in any of the reactions in this illustrative embodiment. Rather, these unsealed areas are provided in space between the working blisters and channels. As compared to pouches that are sealed in all unused space, it has been found that fewer leaks result when unsealed areas such as **255** and **256** are provided, presumably by reducing problematic wrinkles in the film material. Such unsealed areas may be provided on any pouch embodiment.

FIG. **8** shows an illustrative apparatus **800** that could be used with pouch **210**. Instrument **800** includes a support member **802** that could form a wall of a casing or be mounted within a casing. Instrument **800** also includes a second support member **804** that is movable with respect to support member **802**, to allow insertion and withdrawal of pouch **210**. Movable support member **804** may be mounted on a track or may be moved relative to support member **802** in any of a variety of ways. Illustratively, a lid **805** fits over pouch **210** once pouch **210** has been inserted into instrument **800**.

Illustratively, the bladder assembly **810** and pneumatic valve assembly **808** are mounted on movable member **802**, while the heaters **886** and **888** are mounted on support member **802**. However, it is understood that this arrangement is illustrative only and that other arrangements are possible. As bladder assembly **810** and pneumatic valve assembly **808** are mounted on movable support member **804**, these pneumatic actuators may be moved toward pouch **210**, such that the pneumatic actuators are placed in contact with pouch **210**. When pouch **210** is inserted into instrument **800** and movable support member **804** is moved toward support member **802**, the various blisters of pouch **210** are in a position adjacent to the various pneumatic bladders of bladder assembly **810** and the various pneumatic pistons of pneumatic valve assembly **808**, such that activation of the pneumatic actuators may force liquid from one or more of the blisters of pouch **210** or may form pinch valves with one or more channels of pouch **210**. The relationship between the blisters and channels of pouch **210** and the pneumatic actuators of bladder assembly **810** and pneumatic valve assembly **808** are discussed in more detail below with respect to FIGS. **9** and **10**.

Each pneumatic actuator has one or more pneumatic fittings. For example, bladder **824** of bladder assembly **810** has pneumatic fitting **824***a* and pneumatic piston **843** has its associated pneumatic fitting **843***a*. In the illustrative embodiment, each of the pneumatic fittings of bladder assembly **810** extends through a passageway **816** in movable support member **804**, where a hose **878** connects each pneumatic fitting to compressed air source **895** via valves **899**. In the illustrative embodiment, the passageways **816** not only provide access to compressed air source **895**, but the passageways also aid in aligning the various components of bladder assembly **810**, so that the bladders align properly with the blisters of pouch **210**.

Similarly, pneumatic valve assembly **808** is also mounted on movable support member **804**, although it is understood that other configurations are possible. In the illustrative embodiment, pins **858** on pneumatic valve assembly **808** mount in mounting openings **859** on movable support member **804**, and pneumatic pistons **843**, **852**, **853**, and **862** extend through passageways **816** in movable support member **804**, to contact pouch **210**. As illustrated, bladder assembly is mounted on a first side **811** of movable support member **804** while pneumatic valve assembly **808** is mounted on a second side **812** of movable support member **804**. However, because pneumatic pistons **843**, **852**, **853**, and **862** extend through passageways **816**, the pneumatic pistons of pneumatic valve assembly **808** and the pneumatic bladders of bladder assembly **810** work together to provide the necessary pneumatic actuators for pouch **210**.

As discussed above, each of the pneumatic actuators of bladder assembly **810** and pneumatic valve assembly **808** has an associated pneumatic fitting. While only several hoses **878** are shown in FIG. **8**, it is understood that each pneumatic fitting is connected via a hose **878** to the compressed gas source **895**. Compressed gas source **895** may be a compressor, or, alternatively, compressed gas source **895** may be a compressed gas cylinder, such as a carbon dioxide cylinder. Compressed gas cylinders are particularly useful if portability is desired. Other sources of compressed gas are within the scope of this invention.

Several other components of instrument **810** are also connected to compressed gas source **895**. Magnet **850**, which is mounted on a first side **813** of support member **802**, is illustratively deployed and retracted using gas from compressed gas source **895** via hose **878**, although other methods of moving magnet **850** are known in the art. Magnet **850** sits in recess **851** in support member **802**. It is understood that recess **851** can be a passageway through support member **802**, so that magnet **850** can contact blister **246** of pouch **210**. However, depending on the material of support member **802**, it is understood that recess **851** need not extend all the way through support member **802**, as long as when magnet **850** is deployed, magnet **850** is close enough to provide a sufficient magnetic field at blister **246**, and when magnet **850** is retracted, magnet **850** does not significantly affect any magnetic beads present in blister **246**. While reference is made to retracting magnet **850**, it is understood that an electromagnet may be used and the electromagnet may be activated and inactivated by controlling flow of electricity through the electromagnet. Thus, while this specification discusses withdraw-

ing or retracting the magnet, it is understood that these terms are broad enough to incorporate other ways of withdrawing the magnetic field.

The various pneumatic pistons **868** of pneumatic piston array **869**, which is mounted on support **802**, are also connected to compressed gas source **895** via hoses **878**. While only two hoses **878** are shown connecting pneumatic pistons **868** to compressed gas source **895**, it is understood that each of the pneumatic pistons **868** are connected to compressed gas source **895**. Twelve pneumatic pistons **868** are shown. When the pouch **210** is inserted into instrument **800**, the twelve pneumatic pistons **868** are positioned to activate their respective twelve plungers **268** of pouch **210**. When lid **805** is closed over pouch **210**, a lip **806** on lid **805** provides a support for fitment **290**, so that as the pneumatic pistons **868** are activated, lid **805** holds fitment **290** in place. It is understood that other supports for fitment **290** are within the scope of this invention.

A pair of heating/cooling devices, illustratively Peltier heaters, are mounted on a second side **814** of support **802**. First-stage heater **886** is positioned to heat and cool the contents of blister **264** for first-stage PCR. Second-stage heater **888** is positioned to heat and cool the contents of second-stage blisters **282** and **283** of pouch **210**, for second-stage PCR. It is understood, however, that these heaters could also be used for other heating purposes, and that other heaters may be included, as appropriate for the particular application.

If desired, a feedback mechanism (not shown) may be included in instrument **800** for providing feedback regarding whether the sample has actually been forced into a particular blister. Illustrative feedback mechanisms include temperature or pressure sensors or optical detectors, particularly if a fluorescent or colored dye is included. Such feedback mechanisms illustratively may be mounted on either of support members **802** or **804**. For example, a pressure sensor may be mounted on support **802** adjacent the location of blister **264**. When the sample is supposedly moved to blister **264**, if the pressure sensor is depressed, then sample processing is allowed to continue. However, if the pressure sensor is not depressed, then sample processing may be stopped, or an error message may be displayed on screen **892**. Any combination or all of the blisters may have feedback mechanisms to provide feedback regarding proper movement of the sample through the pouch.

When fluorescent detection is desired, an optical array **890** may be provided. As shown in FIG. **8**, optical array **890** includes a light source **898**, illustratively a filtered LED light source, filtered white light, or laser illumination, and a camera **896**. A window **897** through movable support **804** provides optical array **890** with access to second-stage amplification zone **280** of pouch **210**. Camera **896** illustratively has a plurality of photodetectors each corresponding to a second-stage blister **282**, **823** in pouch **210**. Alternatively, camera **896** may take images that contain all of the second-stage blisters **282**, **283**, and the image may be divided into separate fields corresponding to each of the second-stage blisters **282**, **283**. Depending on the configuration, optical array **890** may be stationary, or optical array **890** may be placed on movers attached to one or more motors and moved to obtain signals from each individual second-stage blister **282**, **283**. It is understood that other arrangements are possible.

As shown, a computer **894** controls valves **899** of compressed air source **895**, and thus controls all of the pneumatics of instrument **800**. Computer **894** also controls heaters **886** and **888**, and optical array **890**. Each of these components is connected electrically, illustratively via cables **891**, although other physical or wireless connections are within the scope of this invention. It is understood that computer **894** may be housed within instrument **890** or may be external to instrument **890**. Further, computer **894** may include built-in circuit boards that control some or all of the components, and may also include an external computer, such as a desktop or laptop PC, to receive and display data from the optical array. An interface **893**, illustratively a keyboard interface, may be provided including keys for inputting information and variables such as temperatures, cycle times, etc. Illustratively, a display **892** is also provided. Display **892** may be an LED, LCD, or other such display, for example.

FIG. **9** shows the relationship between bladder assembly **810** and pouch **210** during operation of instrument **800**. Bladder assembly comprises sub-assemblies **815**, **817**, **818**, **819**, and **822**. Because bladder **809** of bladder sub-assembly **815** is large, bladder sub-assembly **815** illustratively has two pneumatic fittings **815***a* and **815***b*. Bladder **809** is used to close off chambers **292** (as shown in FIG. **6**) from the plastic film portion **217** of pouch **210**. When one of the plungers **268** is depressed, one or both of pneumatic fittings **815***a* and **815***b* permit bladder **809** to deflate. After the fluid from one of the chambers **292** passes through, bladder **809** is re-pressurized, sealing off channels **214**, **236**, **245**, **247**, and **249**. While illustrative bladder sub-assembly **815** has only one bladder **809**, it is understood that other configurations are possible, illustratively where each of channels **214**, **236**, **245**, **247**, and **249** has its own associated bladder or pneumatic piston. Bladder sub-assembly **822** illustratively comprises three bladders **824**, **826**, and **828**. As discussed above, bladders **824**, **824**, and **828** drive the three-lobed blister **222** for cell lysis. As illustrated, bladders **824**, **826**, and **828** are slightly larger than their corresponding blisters **224**, **226**, **228**. It has been found that, upon inflation, the surface of the bladders can become somewhat dome-shaped, and using slightly oversized bladders allows for good contact over the entire surface of the corresponding blister, enabling more uniform pressure and better evacuation of the blister. Bladder sub-assembly **817** has four bladders. Bladder **836** functions as a pinch-valve for channel **236**, while bladders **844**, **848**, and **866** are configured to provide pressure on blisters **244**, **248**, and **266**, respectively. Bladder sub-assembly **818** has two bladders **846** and **864**, which are configured to provide pressure on blisters **246** and **264**, respectively. Finally, bladder sub-assembly **819** controls second-stage amplification zone **280**. Bladder **865** acts as a pinch valve for channels **265** and **267**, while bladder **882** provides gentle pressure to second-stage blisters **282** and **283**, to force second-stage blisters into close contact with heater **888**. While bladder assembly **810** is provided with five sub-assemblies, it is understood that this configuration is illustrative only and that any number of sub-assemblies could be used or that bladder assembly **810** could be provided as a single integral assembly.

FIG. **10** similarly shows the relationship between pneumatic valve assembly **808** and pouch **210** during operation of instrument **800**. Rather than bladders, pneumatic valve assembly **808** has four pneumatic pistons **842**, **852**, **853**, and **862**. These pneumatic pistons **842**, **852**, **853**, and **862**, each driven by compressed air, provide directed pressure on channels **242**, **252**, **253**, and **262**. Because the pistons are fairly narrow in diameter, they can fit between bladder sub-assembly **817** and bladder sub-assembly **818** to provide pinch valves for channels **242**, **252**, **253**, and **262**, allowing channels **242**, **252**, **253**, and **262** to be fairly short. However, if desired, pneumatic pistons **842**, **852**, **853**, and **862** could be replaced by bladders, which may be included in bladder assembly **810**, obviating the need for pneumatic valve assembly **808**. It is understood that any combination of bladders and pneumatic

pistons are within the scope of this invention. It is also understood that other methods of providing pressure on the channels and blisters of pouch **210**, as are known in the art, are within the scope of this invention.

EXAMPLE 1

Nested Multiplex PCR

A set of reactions was run in a pouch **110** of FIG. **5**, on an instrument similar to instrument **800** but configured for pouch **110**. To show cell lysis and effectiveness of the two-stage nucleic acid amplification, 50 μL each of a live culture of *S. cerevisaie* and *S. pombe* at log phase was mixed with 100 μL of a nasopharyngeal aspirate sample from a healthy donor to form the sample, then mixed with 200 μL lysis buffer (6M guanidine-HCl, 15% TritonX 100, 3M sodium acetate. 300 μL of the 400 μL sample in lysis buffer was then injected into chamber **192***a* of pouch **110**.

The pouch **110** was manufactured with 0.25 g ZS beads sealed in three-lobed blister **122**. Second-stage primers, as discussed below, were also spotted in blisters **181** and **182** during manufacture of pouch **110**. The pouch **110** was loaded as follows:

115*a* sample and lysis buffer, as described above,
115*b* magnetic beads in the lysis buffer,
115*d-e* wash buffer (10 mM sodium citrate),
115*g* elution buffer (10 mM Tris, 0.1 mM EDTA)
115*h* first-stage PCR buffer:
- 0.2 mM dNTPs
- 0.3 μM each primer:
  - Sc1: primers configured for amplifying a portion of the YRA1 nuclear protein that binds to RNA and to MEX67p of *S. cerevisaie*. The primers are configured to amplify across an intron such that amplification of cDNA (mRNA reverse-transcribed via M-MLV) yields a 180 by amplicon.
  - Sc2: primers configured for amplifying a 121 bp region of the cDNA of MRK1 glycogen synthase kinase 3 (GSK-3) homolog of *S. cerevisaie*.
  - Sc3: primers configured for amplifying a 213 bp region of the cDNA of RUB1 ubiquitin-like protein of *S. cerevisaie*.
  - Sp1: primers configured for amplifying a 200 bp region of the cDNA of suc1-cyclin-dependent protein kinase regulatory subunit of *S. pombe*.
  - Sp2: primers configured for amplifying a 180 bp region of the cDNA of sec14-cytosolic factor family of *S. pombe*.
- PCR buffer with 3 mM $MgCl_2$ (without BSA)
- 50 units M-MLV
- 4.5 units Taq:Antibody
- 100 units RNAseOut

115*j-k* second-stage PCR buffer
- 0.2 mM dNTPs
- 1× LC Green® Plus (Idaho Technology)
- PCR buffer with 2 mM $MgCl_2$ (with BSA),
- 4.5 units Taq

115*l* second-stage PCR buffer with a sample of the first-stage amplicons.

During manufacture, second-stage blisters **181** and **182** were spotted with nested second-stage primers. Each blister was spotted with one primer pair in an amount to result in a final concentration of about 0.3 μM once rehydrated with the second-stage PCR buffer. The second-stage nested primers are as follows:

Sc1: primers configured for amplifying an 80 bp fragment of the Sc1 cDNA first-stage amplicon.
Sc2: primers configured for amplifying a 121 bp fragment of the Sc1 cDNA first-stage amplicon.
Sc3: primers configured for amplifying a 93 bp portion of the Sc1 cDNA first-stage amplicon.
Sp1: primers configured for amplifying a 99 bp portion of the Sc1 cDNA first-stage amplicon.
Sp2: primers configured for amplifying a 96 by portion of the Sc1 cDNA first-stage amplicon.

There is no overlap between the first-stage and second stage primer pairs for any of the targets. Each pair of primers was spotted into one negative control blister **181** and two second-stage blisters **182**, so that each second-stage amplification would be run in duplicate, each duplicate with a negative control.

After loading, activation of the plunger associated with entry channel **115***a* moved the sample to three-lobed blister **122**, activation of the plunger associated with entry channel **115***b* moved the magnetic beads to reservoir **101**, activation of the plungers associated with entry channels **115***d-e* moved wash buffer to reservoirs **102** and **103**, activation of the plunger associated with entry channel **115***g* moved elution buffer to reservoir **104**, activation of the plunger associated with entry channel **115***h* moved first-stage PCR buffer to reservoir **105**, activation of the plungers associated with entry channels **115***j-k* moved second stage PCR buffer to reservoirs **106** and **107**, and activation of the plunger associated with entry channel **115***l* moved the positive control (second-stage PCR buffer with a sample of previously prepared first-stage amplicon) to reservoir **108**. In this present example, the plungers associated with entry channels **115***a* and **115***b* were depressed prior to loading the pouch **110** into the instrument. All other plungers were depressed sequentially in the instrument during the run, and fluids were moved to reservoirs **102** through **108** as needed.

Once pouch **110** was placed into the instrument, and beating took place for ten minutes in the presence of ZS beads, as described above. Once cell lysis was complete, reservoir **101** was compressed and nucleic acid binding magnetic beads from reservoir **101** were forced into three-lobed blister **122**, where the beads were mixed gently and allowed to incubate for 5 minutes.

The sample-bead mixture was then moved to blister **144**, where the magnetic beads were captured via activation of the magnet. Once the magnet was deployed, bladders adjacent blister **144** were pressurized to force fluids back to three-lobed blister **122**. The captured beads were then washed as described above, using the wash solution from reservoirs **102** and **103**. Following washing, the beads were once again captured in blister **144** via activation of the magnet, and the elution buffer stored in reservoir **104** is moved to blister **144**, where, after a 2 minute incubation, the nucleic acids eluted from the beads are then moved to blister **161**, as discussed above.

In blister **161**, the nucleic acid sample is mixed with first-stage PCR master mix from reservoir **105**. The sample is then held at 40° C. for 10 minutes (during which time M-MLV converts mRNA to cDNA), then 94° C. for 2 minutes (to inactivate the M-MLV and remove antibody from taq). Thermal cycling is then 20 cycles of 94° C. for 10 second and 65° C. for 20 seconds.

Subsequent to first-stage amplification, the sample is diluted approximately 100-fold using the second-stage PCR master mix from reservoir **106**. The sample is then moved to blisters **182**, which were previously spotted with the second-stage primers, as discussed above. Second-stage PCR buffer

was moved from reservoir **181** to negative control blisters **181**, and the positive control mixture was moved to blisters **183** from reservoir **108**. The samples were denatured for 30 seconds at 94° C., then amplified for 45 cycles of 94° C. for 5 seconds and 69° C. for 20 seconds.

As can be seen in FIG. **13**, all target amplicons and the positive control showed amplification, while none of the negative controls showed amplification. Each sample was run in replicates. The replicates each showed similar amplification (data not shown).

It is understood that the *S. cerevisaie* and *S. pombe* targets are illustrative only and that other targets are within the scope of this invention.

EXAMPLE 2

iPCR

In another example, the pouches and instruments of the present invention may be used for immuno-PCR (iPCR). iPCR combines the antibody specificity of ELISA with the sensitivity and multiplex capabilities of PCR. While iPCR has been applied to diagnostics and toxin detection, iPCR has not enjoyed widespread commercial application, presumably because PCR template contamination issues are severe in an open ELISA format. Because the pouch format of the present invention provides a sealed environment, the pouches of the present invention may be well suited for iPCR.

A traditional ELISA detection scheme is shown in FIG. **11** (labeled "ELISA"). In 1992, Cantor and colleagues (Sano, T., et al, Science, 1992. 258(5079): p. 120-2, herein incorporated by reference) described a modification of the basic ELISA technique (FIG. **11**, similar to the "Immuno-PCR I" scheme without capture antibody C-Ab), in which the enzyme used for generating a specific signal is replaced by a unique DNA fragment indirectly attached to the reporter antibody R through a bi-functional binding moiety S, such as a streptavidin-protein A chimera. The DNA fragment is subsequently detected by PCR. It is known that PCR detection can provide dramatic increases in immuno-PCR assay sensitivity over corresponding ELISA assays, with improvements to sensitivity commonly 10^2 to 10^4-fold. Advances in quantitative real-time PCR methods have improved the speed and quantification of immuno-PCR. Direct coupling of the reporter antibody (R-Ab) with DNA template tags (FIG. **11**, "Immuno-PCR II" scheme) has further increased the assay sensitivity 10^2 to 10^3 fold and made possible the development of multiplex immuno-PCR assays, in which each different antibody is tagged with a different oligonucleotide and, thus, each antigen is associated with a unique amplification product.

Despite these advantages over traditional ELISAs, iPCR has not been widely adopted in commercial products in the 13 years since it was first described. This is due in part to the contamination hazards inherent in any open-tube PCR analysis method. Prior art iPCR protocols are derived from ELISA assays and require numerous wash steps that increase the likelihood of contaminating the work area with amplified material. The significant risk of false positives due to workflow contamination has contributed to the avoidance of iPCR in diagnostic assessment.

Amplicon contamination issues slowed the widespread adoption of PCR itself in the diagnosis of human genetic conditions or of infectious disease until homogenous (i.e. "closed-tube") PCR assays were developed. By making the readout of the assay possible in a closed-tube system, spread of amplicon is severely curtailed. Similarly, iPCR may be more widely adopted if a closed system format were available. In the present system, the sample would be injected into a pouch that would be provided with all required reagents. The steps of antigen capture, wash, reporter-antibody binding, wash, and subsequent PCR detection could be performed completely within the pouch. Illustratively, nucleic acids would never leave the pouch and would be disposed of along with the pouch.

Any of the pouches of the present invention may be adapted for iPCR. For example, the pouch **210** of FIG. **6** illustratively may be adapted as follows. Chambers **292***a* through **292***l* would be filled with the following components. The sample illustratively comprising an unpurified and/or unmodified antigen (e.g. a toxin) is injected through injection port **241***a* to chamber **292***a*. A capture antibody conjugated to magnetic beads (C-Ab) is provided in chamber **292***b*. If multiple targets are to be tested, it is understood that multiple capture antibodies having specificity for multiple antigens may be used. An optional pre-wash buffer is provided in chamber **292***c*. A reporter antibody conjugated to an oligonucleotide template (R-Ab-DNA) is provided in chamber **292***d*. It is understood that the capture and reporter antibodies may be monoclonal or polyclonal. When multiple antigens are to be detected, the capture and reporter antibodies may contain only polyclonal antibodies, only monoclonal antibodies, or any combination of polyclonals specific for one antigen and monoclonals specific for another antigen. When a reporter antibody is polyclonal, it is understood that all reporter antibodies having specificity for a particular antigen will be coupled to oligonucleotide templates have one specific sequence, even if the specificity between various antibodies in that set varies. The oligonucleotide may be double-stranded or single-stranded. Multiple R-Ab-DNAs may be provided to detect multiple antigens, with each different antibody conjugated to a unique oligonucleotide. Wash buffers are provided in chambers **292***e* through **292***h*. A first-stage PCR master mix, as described above, is provided in chamber **292***i*. A dilution buffer is provided in chamber **292***j*. A second-stage PCR master mix, as described above, is provided in chamber **292***k*. As discussed above, the reagents may be provided dried in chambers **292***b* through **292***l*, and may be rehydrated prior to use via injection of water through seal **239**, or each reagent may be provided wet via injection to each individual chamber **292**. Combinations thereof are contemplated.

Once the sample and reagents are loaded, the pouch **220** is inserted into the instrument **800**. Plunger **268***a* is then depressed and the sample is moved to three-lobed blister **222**. Plunger **268***b* is also depressed and the capture antibodies conjugated to magnetic beads (shown as C in FIG. **11**) are also moved to three-lobed blister **222**. The sample and the C-Ab are mixed via pressure from bladder **828** alternating with pressure from bladders **824**, **826**. Because mixing is desired, pressure from the bladders **824**, **826**, **828** may be considerably lower than the pressure used as discussed above for lysis. Illustratively, gentle mixing is obtained. The sample and the C-Ab are allowed to incubate for sufficient time for the capture antibodies to bind to antigens T in the sample (forming C-Ab-T complexes), illustratively for about 5 minutes, although other incubation times may be desirable. For iPCR, it may be desirable to include an additional heater in instrument **800** to maintain incubations at about 37° C.

Once the antigens present in the sample have been sufficiently incubated for capture, the sample is moved to blister **246** and the magnet **850** is deployed, capturing the capture antibodies in blister **246**. The unbound portions of the sample are then moved back to three-lobed blister **222**, which now functions as a waste reservoir. While magnetic beads are used

to restrain the capture antibodies in the examples described herein, it is understood that other capture mechanisms may be used, including solid supports, possibly even cross-linking the capture antibodies to an interior surface of a blister.

If desired, the C-Ab-T complexes may be washed using the pre-wash buffer from chamber **292***c*. Pre-wash buffer is moved into blister **244** via channel **245**, the magnet is withdrawn, releasing the C-Ab-T complexes, and the beads are gently moved between blisters **244** and **246**. The beads are then recaptured in blister **246** via activation of the magnet **850**, and the remaining fluid is moved to three-lobed blister **222**. It is expected that this pre-wash may improve discrimination of a positive signal over the background negative signal, but such differences may prove to be insignificant. Additional pre-washes may be performed, if desired.

Plunger **268***d* is depressed and the mixture containing one or more reporter antibodies R-Ab conjugated to oligonucleotide templates (R-Ab-DNA, shown in FIG. **11**, Immuno-PCR II scheme, as R with attached nucleic acid) is moved to blister **246**. The magnet is retracted and the mixture is gently mixed by moving between blisters **244** and **246**. Incubation, illustratively for about 5 minutes although other incubation times may be desirable, allows formation of the ternary complexes C-Ab-T-R-Ab-DNA, as illustrated in FIG. **11**, Immuno-PCR II. Activation of the magnet **850** allows capture of the ternary complexes in blister **246**, and the remaining fluid is moved to three-lobed blister **222**.

Plunger **268***e* is depressed and wash buffer is moved from chamber **292***e* to blister **246**. The magnetic bead-ternary complex is washed as in the pre-wash described above, the magnetic bead-ternary complex is recaptured in blister **246**, and the remaining fluid is moved to three-lobed blister **222**. Washing is repeated multiple times using the wash buffers from chambers **292***f*, **292***g*, and **292***h*, except that mixing is between blisters **246** and **248** to avoid reintroducing unbound R-Ab-DNA complexes that may be residing in blister **244** or channel **243**. While four washes are described in this illustrative embodiment, it is understood that any number of washes may be used, illustratively by altering the number of chambers in the fitment **290** or by increasing the volume of the chambers and using only a portion of the wash buffer in a chamber for each wash. It is also understood that removal of all unbound R-Ab-DNA complexes is extremely difficult, even with a large number of washes. Further, for an antigen that is not present in the sample, the presence of just a few molecules of unbound R-Ab-DNA or non-specifically bound R-Ab-DNA complexes specific for that antigen may result in an amplification signal. Thus, while the ideal goal of the washing step is to remove all R-Ab-DNA complexes specific for antigens that are not present in the sample, one illustrative goal is to remove a sufficient number of such R-Ab-DNA complexes such that the amplification curve for that oligonucleotide is delayed and can be distinguished from the amplification curve of a positive sample. Illustratively, more washes should remove more unbound R-Ab-DNA and provide for a lower detection limit, but more washes risk loss of desired ternary complexes through dissociation or loss of magnetic beads not captured by the magnet. After washing is complete, if desired, the captured ternary complex may be heated or enzymatically treated (illustratively with papain, proteinase K, or other suitable enzyme provided via an additional chamber) to release the DNA prior to PCR. Such treatment may improve the first-stage PCR efficiency. It is understood that such treatment may be used with any of the iPCR examples discussed herein.

Once washing is complete, plunger **268***i* is depressed and the first-stage PCR master mix, as described above, is moved to blister **246**. First-stage PCR master mix contains primer pairs for all desired targets. The magnet **850** is released, and optional mixing between blisters **246** and **248** may be used to resuspend the ternary complexes. The mixture is moved to blister **264**, where first-stage thermal cycling takes place, as described above. Once the complexed oligonucleotides have been amplified to sufficient levels, as discussed above, the amplified mixture is optionally diluted using the dilution buffer provided in chamber **292***j*. Some or all of the first-stage amplified mixture may be mixed with the second-stage PCR master mix provided from chamber **292***k*, and then this mixture is moved to the 18 second-stage blisters **282**, where second-stage primers are provided, as discussed above. If desired, one of the second-stage blisters **282** may be used for a negative control, wherein it is known that no antigen is present in the sample, but R-Ab-DNA was provided from chamber **292***d* and the proper primers are provided in the negative control second-stage blister **282**. It is expected that, despite various washes, small amounts of this particular R-Ab-DNA may be present in the first-stage PCR and, accordingly, that small amounts of the first-stage amplified product may be provided to this second-stage blister **282**. However, the amounts should be quite small, and the crossing point should be delayed well past that of positive samples. Also, if desired, one of one of the second-stage blisters may be used for a positive control, wherein the sample is spiked with an antigen that is not otherwise being tested (perhaps included with the C-Ab beads), which presumably will bind its corresponding R-Ab-DNA, and which is then amplified in the first-stage PCR. Finally, control blisters **283** are not used in this illustrative embodiment. However, with a minor reconfiguration, blisters **283** may be connected to blister **266** and may provide for six additional second-stage reactions. Alternatively, blisters **283** may be used for other controls, as are desired by the particular application.

As discussed above, because of the difficulty in removing all unbound or non-specifically bound R-Ab-DNA complexes, even negative samples may show some amplification. It is expected that real-time amplification analysis will allow positives to be distinguished from negatives via a difference in cycle number of a threshold crossing point (or an equivalent cycle threshold measurement, such as the cycle number when 50% of amplification is reached).

It is understood that the first-stage multiplex amplification may not be necessary for detection with iPCR, even when testing for multiple antigens. However, the first-stage multiplex amplification may afford more sensitivity.

EXAMPLE 3

iPCR with iPCR-Specific Pouch

The above example illustrates a method adapting the pouch **210** of FIG. **6** for iPCR. However, FIG. **12** shows a pouch **310** that is illustratively configured for iPCR. Fitment **390** is similar to fitments **190** and **290**, except having 15 chambers **392** and plungers **368**. Each chamber **392** (illustratively chamber **392***a*, where the sample is injected) may have its own injection port, or several chambers may have a connecting channel and may share an injection port (illustratively **392***e* through **392***k*, each containing wash buffer). As with the above-described fitments, any combination of injection ports and channels is within the scope of this invention. Pouch **310** differs from pouch **210** of FIG. **6** in one primary way. As cell lysis is usually not needed in iPCR, the three-lobed blister **222** may be replaced by a single large waste reservoir **322**. Because multiple washes are desirable in iPCR, waste reservoir **322** is

provided with a sufficiently large volume to retain the multiple used buffers, for example 2-5 ml, depending on the application and volume of the reactions. It is understood that instrument **800** may need to be reconfigured somewhat to accommodate pouch **390**.

Prior to insertion into the instrument, pouch **390** of FIG. **12** illustratively would have the following components in the chambers **392**. The sample to be tested would be injected into chamber **392***a*. Capture antibodies (C-Ab) conjugated to magnetic beads are provided in chamber **392***b*. An optional pre-wash buffer is provided in chamber **392***c*. Reporter antibodies conjugated to their respective oligonucleotide templates (R-Ab-DNA) are provided in chamber **392***d*. As discussed above, multiple R-Ab-DNAs may be provided to detect multiple antigens, with each different antibody conjugated to a unique oligonucleotide. Wash buffers are provided in chambers **392***e* through **392***k*. A first-stage PCR master mix is provided in chamber **392***l*. A dilution buffer is provided in chambers **392***m* and **392***n*. A second-stage PCR master mix is provided in chamber **392***o*.

To begin, plungers **368***a* and **368***b* are depressed, forcing the sample and the capture antibodies C-Ab through channel **343** into blister **344**. The sample and the C-Ab are gently mixed, illustratively by moving between blisters **344** and **346** via channel **345**, and are incubated as described above. After a sufficient period of time for formation of the C-Ab-T complex, the mixture is moved to blister **346** via channel **338**, where a magnet **350** housed in the instrument is deployed, capturing the complexed beads therein. The remaining fluid is moved to waste reservoir **322**, via channel **339**. Optionally, pre-wash buffer from chamber **392***c* is moved to blister **346** via channel **345**, the magnet **350** is withdrawn, and the magnetic beads are gently washed by moving the fluid between blisters **344** and **346**. The magnet **350** is again deployed and the beads are again captured in blister **346**.

Next, plunger **368***d* is depressed moving the reporter antibodies conjugated to nucleic acid template (R-Ab-DNA) to blister **346**, the magnet **350** is withdrawn, and the C-Ab-T and the R-Ab-DNA are gently mixed illustratively by moving between blisters **344** and **346** via channel **345** and are incubated as described above. After formation of the ternary complex (C-Ab-T-R-Ab-DNA), the magnet **350** is once again deployed, capturing the ternary complex in blister **346**, and the remaining fluid is moved to waste blister **322**.

The ternary complex is then washed using the wash buffer from chamber **392***e*, as described above for the pre-wash. The magnet **350** is again deployed, capturing the ternary complex in blister **346**, and the remaining fluid is moved to waste blister **322**. Washing is repeated various times, using the wash buffer from chambers **392***f* through **392***k*. Thus, in the illustrative embodiment of FIG. **12**, seven washes are completed. However, as discussed above, more or fewer washes may be desirable, depending on the particular application.

As illustrated in the Immuno-PCR II scheme shown in FIG. **11**, the reporter antibody is conjugated directly to the nucleic acid template. It is understood that the reporter antibody in any of the embodiments discussed herein could be attached to the nucleic acid template by any of a variety of ways, including direct and indirect covalent and non-covalent bonding. Also, the reporter antibody could be attached to the nucleic acid through a variety of mechanisms, including, for example, through the use of secondary antibodies, as illustrated in the Immuno-PCR I scheme of FIG. **11**. If secondary antibodies or other indirect coupling mechanisms are used, it may be desirable to add additional ports and further washing steps.

The first-stage PCR master mix, as described above, is then deployed to blister **346** via activation of plunger **368***k*, and the magnet **350** is once again withdrawn. If gentle mixing is desired, the fluid may be moved between blisters **346** and **364** via channel **347**. While mixing can take place between blisters **346** and **344** as before, in the illustrative embodiment mixing takes place between blisters **346** and **364**. This aids in reducing the reintroduction of unbound reporter antibody complexes that may be residing in blister **344**. The sample is then moved to blister **364**. A bladder positioned over **364** is gently pressurized to move blister **364** into contact with a heating/cooling device, such as a Peltier device, and the sample would be thermocycled, as discussed above for first-stage PCR. As discussed above in the previous example, first-stage PCR may be unnecessary with the presently described iPCR, blister **364** and its associated heater may be omitted, and all washes illustratively could take place by mixing between blisters **344** and **346**. If first-stage PCR is omitted, the dilution, as discussed below may also be omitted.

Most of the amplified sample is moved to waste blister **322**, leaving some amplified sample behind in blister **364** to be diluted. It is understood that if space constraints or other considerations limit the size of blister **322**, blisters **344** and **346** may be used to contain the remaining waste. The small amount of remaining amplified sample is mixed with dilution buffer from chamber **392***m*, which has been moved to blister **366** via channel **349**. The sample and the dilution buffer may be mixed gently between blisters **364** and **366**, via channel **355**. If further dilution is desired, dilution may be repeated using the dilution buffer from chamber **392***n*. Finally, some of the diluted sample is moved to waste reservoir **322** and the remaining diluted sample is mixed with second-stage PCR master mix from chamber **392***o*. After mixing, the sample is moved to the various low volume second stage blisters **382**, where second-stage primers are provided, as discussed above. In the present configuration, blister **383** may be used for a negative control and blister **384** may be used for a positive control, as discussed above in the previous iPCR example. Second-stage PCR and analysis takes place as described above in the previous iPCR example.

EXAMPLE 4

Combined PCR and iPCR

In some circumstances, it may be desirable to test for antigens and nucleic acids in one reaction set. For example, a terrorist attack may employ various agents to kill multiple people. In responding to the attack, it may be unknown if the causative agent is a virus, bacterium, or other organism, or if the causative agent is a toxin. The closed-environment system of the pouches of the present invention is well suited for such use. In the embodiment disclosed herein, both PCR and iPCR may take place within a single pouch, allowing for simultaneous detection of various biological and antigenic agents.

FIG. **13** shows a pouch **410** that is similar to pouch **210** of FIG. **6**. Illustrative pouch **410** has all of the blisters of pouch **210**, but also includes blisters **430**, **431**, **432**, and **433**. Pouch **410** also has a larger fitment **490**, having twenty chambers **492** with twenty corresponding plungers **468**. As above, the fitment could include separate injection ports for each chamber, or various chambers could have connecting channels. Various combinations thereof are within the scope of this invention. The instrument for pouch **410** would be similar to instrument **800**, except that additional pneumatic actuators would be needed for blisters **430**, **431**, **432**, and **433** and

channels **436**, **457**, **473**, **486**, **487**, and **488**, as well as two additional retractable magnets **451** and **454** adjacent blisters **433** and **431**, respectively.

In the illustrative embodiment, the chambers would be loaded as follows. iPCR wash buffer would be provided in chambers **492***a* through **492***e* and **492***j*. The sample to be tested would be injected into chamber **492***f*. The capture antibodies (C-Ab) conjugated to magnetic beads are provided in chamber **492***g*. An optional pre-wash buffer is provided in chamber **492***h*. Reporter antibodies conjugated to their respective oligonucleotide template (R-Ab-DNA) are provided in chamber **492***i*. A cell lysis buffer is provided in chamber **492***k*. Nucleic-acid-binding magnetic beads are provided in chamber **492***l*. Nucleic acid wash buffers are provided in chambers **492***m* and **492***n*. A nucleic acid elution buffer is provided in chamber **492***o*. A first-stage PCR master mix is provided in chamber **492***p*. A dilution buffer is provided in chambers **492***q* and **492***r*. A second-stage PCR master mix is provided in chamber **492***s*. Controls, as discussed above with respect to FIG. **6**, are provided in chamber **492***t*. It is understood that this arrangement is illustrative and that other configurations are possible. Also, as with the other examples discussed above, one or more of these components may be provided dried in one or more of the blisters of pouch **410**.

Once the sample is loaded into chamber **492***f* and pouch **410** is loaded into the instrument, plungers **468***f* and **468***g* are depressed, moving the sample and C-Ab through channel **436** to blister **430**. The sample and capture antibodies may be mixed by gently moving them between blisters **430** and **431** and then incubated as described above, to encourage formation of C-Ab-T complexes. The sample is moved to blister **431** and magnet **454** is activated, capturing the C-Ab-T complexes therein. Thus, toxins or other targeted antigens are now captured in blister **431**. It is noted that, in the illustrative embodiment, the surface of the magnetic bead portion of the magnetic beads coupled to the capture antibodies is different from the surface of the nucleic-acid-binding magnetic beads, and the magnetic beads coupled to the capture antibodies is illustratively configured not to bind nucleic acids. The remaining fluid is then moved to three-lobed blister **422** via channel **473**. This fluid can then be processed and assayed for the presence of target nucleic acids. This division of the sample may be problematic if a targeted antigen is a surface antigen of an organism targeted in the PCR detection. In such a situation, it may be desirable to choose between antigen detection and nucleic acid detection for that organism, or to use separate pouches for PCR and iPCR. Alternatively, the sample may be lysed prior to antibody capture. If lysis would interfere with antibody capture, for example by changing the conformation of the antigen, then the sample may be divided and just a portion of the sample may be lysed prior to antibody capture. If a pre-wash of the C-Ab-T is desired, plunger **468***h* is activated and the pre-wash buffer from chamber **492***h* is moved into blister **431**. Magnet **454** is withdrawn, the fluid is mixed between blisters **430** and **431**, and magnet **454** is once again deployed, capturing the C-Ab-T complex in blister **431**. The wash buffer, now possibly containing cells that had been left behind after capture, is moved to three-lobed blister **422**, along with the rest of the uncaptured material.

It is understood that the sample is now divided into two parts for separate processing. Antigens present in the sample are now captured in C-Ab-T complexes in blister **431**, while cells, viruses, and free nucleic acids present in the sample are now in three-lobed blister **422** awaiting lysis. The two portions of the sample are processed separately until both are ready for first-stage PCR. These processes may take place in any order or simultaneously. However, in the present embodiment, cell lysis must take place prior to substantial processing of the C-Ab-T complexes, so that three-lobed blister may then function as the waste reservoir. If a separate waste reservoir is used, cell lysis can be delayed until after the C-Ab-T complexes have been processed, if desired.

Lysis buffer from chamber **492***k* is moved into three-lobed blister **422** via channel **436**. Bladders adjacent the blisters of three-lobed blister **422** are pressurized as described above with respect to FIG. **6**, driving high velocity collisions, shearing the sample, and liberating nucleic acids. Once the cells have been adequately lysed, plunger **468***l* is activated and nucleic acid binding magnetic beads stored in chamber **492***l* are injected via channel **436** into three-lobed blister **220**. The sample is mixed with the magnetic beads and the mixture is allowed to incubate. The processing then continues as described above with respect to the pouch of FIG. **6**. The mixture of sample and beads are forced through channel **438** into blister **444**, then through channel **443** and into blister **446**, where a retractable magnet **450** captures the magnetic beads from the solution. The un-captured liquid is then forced out of blister **446** and back through blister **444** and into blister **422**, which is now used as a waste receptacle. Plunger **468***m* may be activated to provide a wash solution to blister **444** via channel **445**, and then to blister **446** via channel **447**. Magnet **450** is retracted and the magnetic beads are washed by moving the beads back and forth from blisters **444** and **446**. Once the magnetic beads are washed, the magnetic beads are recaptured in blister **446** by activation of magnet **450**, and the wash solution is then moved to blister **422**. This process may be repeated using wash reagents in chambers **492***n*. However, it is understood that more or fewer washes are within the scope of this invention. After washing, elution buffer stored in chamber **492***o* is moved via channel **447** to blister **448**, and the magnet **450** is retracted. The solution is cycled between blisters **446** and **448** via channel **452**, breaking up the pellet of magnetic beads in blister **446** and allowing the captured nucleic acids to come into solution. The magnet **450** is once again activated, capturing the magnetic beads in blister **246**, and the eluted nucleic acid solution is moved into blister **448**.

Returning back to blister **431**, the C-Ab-T complexes are therein captured. Plunger **468***i* is depressed and the reporter antibodies conjugated to nucleic acid template (R-Ab-DNA) are introduced to blister **430**, the magnet **454** is withdrawn, and the C-Ab-T and the R-Ab-DNA are gently mixed, illustratively by moving between blisters **430** and **431** via channel **457**, and are incubated as described above. After formation of the ternary complex (C-Ab-T-R-Ab-DNA), magnet **454** is once again deployed, capturing the ternary complex in blister **431**, and the remaining fluid is moved to blister **422**, which is now used as a waste reservoir.

The ternary complex is then washed using the wash buffer from chamber **492***j*, as described above for the pre-wash. Magnet **454** is again deployed, capturing the ternary complex in blister **446**, and the remaining fluid is moved to blister **422**. Additional wash buffer from chamber **492***a* is injected into blister **432** via channel **486**, the magnet **454** is withdrawn, and the ternary complex is resuspended by mixing the fluids blisters **431** and **432**. The fluids are then moved to blister **433** via channel **487** and the ternary complex is captured therein via activation of magnet **451**. The waste fluids are then moved back through blisters **433** and **432** to blister **422**. Additional wash buffer is introduced into blister **432** from chamber **492***b* and washing is repeated by mixing between blisters **432** and **433**. Washing is repeated various times using the wash buffer from chambers **492***c* through **492***e*. Thus, in the illustrative embodiment of FIG. **13**, six washes are completed. However,

as discussed above, more or fewer washes may be desirable, depending on the particular application. It is understood that blisters **432** and **433** are used to minimize contamination from prior washes. If desired, blisters **432** and **433** may be omitted and the wash buffers contained in chambers **492***a* through **492***e* may be provided directly to either blister **430** or **431**, with mixing between blisters **430** and **431**.

The washed antibody ternary complex is now captured in blister **433** and the eluted nucleic acids are now in blister **448**. It is noted that the antibody ternary complex and the eluted nucleic acids may be processed through PCR in independent reactions, through to separate sets of second-stage PCR blisters. However, in the present embodiment the antibody ternary complex and the eluted nucleic acids are combined for PCR analysis. First-stage PCR master mix, containing all first-stage primers, is injected from chamber **492***p* into blister **448**. The nucleic acid sample is then mixed between blisters **448** and **464** via channel **453**. If first-stage PCR is desired for the iPCR components, the nucleic acid sample is then moved to blister **433**, magnet **451** is withdrawn, and the re-united sample is illustratively mixed between blisters **433** and **464**. The sample is then moved to blister **464**, where the sample is thermocycled, as discussed above. Next, the amplified sample may be diluted once or several times, using the dilution buffers from chambers **492***q* and **492***r*. Prior to each dilution, a large portion of the amplified sample is removed from blister **464** via either channel **447** or channel **488**. With each addition of dilution buffer, the sample is mixed between blisters **464** and **466** via channel **462**. After dilution, all or a portion of the sample is mixed with the second-stage PCR master mix from chamber **492***s*, as described in the examples above.

The sample is then moved from blister **466** via channel **465** to blisters **482** in second-stage amplification zone **480**. Blisters **482** each had been previously provided with a primer pair, some of the primer pairs specific for target nucleic acids, while other primer pairs specific for an oligonucleotide conjugated to a reporter antibody. If desired, two blisters **482** may be dedicated to iPCR controls, as discussed above. Blisters **483** may be used for PCR controls, as discussed above with respect to blisters **283** of FIG. **6**. While **18** blisters **482** are shown, it is understood that any number of blisters **482** may be used. Second-stage PCR amplification proceeds as discussed above with respect to FIG. **6**. It is understood that PCR analysis may use amplification curves, melting curves, or a combination thereof, while iPCR analysis may use crossing thresholds, as discussed above. Other methods of analysis are within the scope of this invention.

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While references are made herein to PCR and iPCR, it is understood that the devices and methods disclosed herein may be suitable for use with other nucleic acid amplification or other biological processing methods, as are known in the art, particularly methods that benefit from a first-stage multiplex reaction and a second-stage individual reaction. Illustrative non-limiting second-stage reactions include primer extension, including allele-specific primer extension; extension terminations, including termination by incorporation of one or more dideoxy nucleotides; incorporation of fluorescent or non-fluorescent labels; and other enzymatic reactions requiring a change in reaction mixture components or component ratios, such as asymmetric PCR, allele-specific PCR, invader assays, and other isothermal amplification or detection chemistries.

Although the invention has been described in detail with reference to preferred embodiments, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.

The invention claimed is:

1. A container for performing two-stage nucleic acid amplification on a sample in a closed system comprising

one or more sealable ports, the sealable ports providing the only access from an exterior of the container such that when all of the one or more sealable ports is sealed, the container is fully closed, the one or more sealable ports including a sample injection port for introducing the sample into the container,

a cell lysis zone configured for lysing cells or spores located in the sample, the cell lysis zone fluidly connected to the sample injection port,

a nucleic acid preparation zone fluidly connected to the cell lysis zone, the nucleic acid preparation zone configured for purifying nucleic acids,

a first-stage reaction zone fluidly connected to the nucleic acid preparation zone, the first-stage reaction zone comprising a first-stage reaction blister configured for first-stage amplification of the sample, and

a second-stage reaction zone fluidly connected to the first-stage reaction zone, the second-stage reaction zone comprising a plurality of second-stage reaction chambers, each second-stage reaction chamber comprising a pair of primers configured for further amplification of the sample, the second-stage reaction zone configured for contemporaneous thermal cycling of all of the plurality of second-stage reaction chambers.

2. The container of claim **1** wherein the first-stage amplification is multiplex amplification for a plurality targets, and each pair of primers in the second-stage reaction chambers is configured for further amplification of one of the first-stage targets.

3. The container of claim **2** wherein each pair of primers in the second stage reaction chamber is configured to be nested within primers used in amplifying the first-stage targets.

4. The container of claim **1** wherein the one or more sealable ports further comprise a second port configured to receive water or reagents, and the sealable ports are configured to be sealed subsequent to introduction of the sample and water or reagents and remain sealed through first- and second-stage amplification.

5. The container of claim **1** wherein the cell lysis zone comprises a cell lysis blister containing a plurality of lysing particles.

6. The container of claim **5** wherein the particles are zirconium silicate beads.

7. The container of claim **6** wherein the nucleic acid preparation zone comprises DNA-binding magnetic beads.

8. The container of claim **1** wherein the blister comprises a flexible material, such that pressure provided on the blister collapses the blister, forcing the contents from the blister.

9. The container of claim **8** wherein the flexible material is a flexible plastic film.

10. The container of claim **1** wherein the second-stage reaction zone further comprises one or more additional second-stage reaction chambers, each of the additional second-stage reaction chambers configured as amplification controls.

11. The container of claim **1** wherein the sealable ports are heat sealable.

12. A container for performing two-stage nucleic acid amplification on a sample in a closed system comprising

one or more sealable ports, the sealable ports providing the only access from an exterior of the container such that when all of the one or more sealable ports is sealed, the container is fully closed, the one or more sealable ports including a sample injection port for introducing the sample into the container,

a first-stage reaction zone fluidly connected to the sample injection port, the first-stage reaction zone comprising a first-stage reaction blister configured for first-stage amplification of the sample, and

a second-stage reaction zone fluidly connected to the first-stage reaction zone, the second-stage reaction zone comprising a plurality of second-stage reaction chambers, each second-stage reaction chamber comprising a pair of primers configured for further amplification of the sample, the second-stage reaction zone configured for contemporaneous thermal cycling of all of the plurality of second-stage reaction chambers.

13. The container of claim **12** further comprising a cell lysis zone configured for lysing cells or spores located in the sample, a nucleic acid preparation zone fluidly connected to the cell lysis zone, the nucleic acid preparation zone configured for purifying nucleic acids, the cell lysis zone and the nucleic acid preparation zone both fluidly located between the sample injection port and the first-stage reaction zone.

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,940,526 B2
APPLICATION NO. : 13/765249
DATED : January 27, 2015
INVENTOR(S) : Ririe et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On Title Page:

Abstract, Item 57, Line 3: Please correct "analyzes include"
to read -- analyses includes --

Signed and Sealed this
Seventh Day of July, 2015

Michelle K. Lee

Michelle K. Lee
Director of the United States Patent and Trademark Office